METHODS IN

MEDICAL RESEARCH

VOLUME I

METHODS IN
Medical Research

VOLUME I

VAN R. POTTER, *Editor-in-Chief*

ASSAY OF ANTIBIOTICS, *Henry Welch, Editor*

CIRCULATION—BLOOD FLOW MEASUREMENT, *Harold D. Green, Editor*

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THE YEAR BOOK PUBLISHERS · INC.

304 SOUTH DEARBORN STREET · CHICAGO

PRINTED IN U.S.A.

PREFACE

It seems to have become customary, in launching a new periodical or set of volumes in the field of medical research and its ancillary sciences, to adopt a faintly apologetic and deprecatory attitude; and it is particularly fitting to do so in the present case, which may be thought rather novel and ambitious both in plan and in objectives and which at best can hardly establish the general usefulness we hope for until several volumes have been distributed.

This series is to be devoted to methods and techniques, and there are four main reasons for our conclusion that such a series may be useful. In the first place, while the results of investigations are constantly subject to critical review, it is not usually easy to find anywhere an appraisal and discussion of the various methods that may have been proposed for the solution of some experimental problem. In the second place, it is becoming difficult, especially in physiology, to obtain publication of a paper dealing solely with a technique or even to include an adequate description of the technique in a paper describing the results obtained. Third, it frequently happens that a method is modified and improved in continued use, either in the laboratory whence it originated or elsewhere; such useful modifications find their way into print, if at all, only as brief and scattered indications and are to a great extent diffused by the uncertain process of personal communication. Fourth, many methods developed during the war have been described only in official reports.

Each volume will be divided into four or five principal, self-contained sections, each of which shall, for that volume, represent one of the broad fields of medical research: biochemistry, physiology and pharmacology, microbiology and immunology, and biophysics including radiobiology. Within each of these broad fields we shall try, year by year, to select narrower topics wherein a restatement of techniques seems timely. For example, the following topics have been considered among many others for inclusion in forthcoming volumes: methods related to acetylcholine; assay of hormones and their excretion products in urine; experimental surgery of the autonomic system; techniques of histochemistry; paper chromatography; design and use of stimulators; methods in the study of pulmonary function; methods in the study of bacterial viruses; and so forth.

When the topics have been selected, we shall try to find experts, like those who have so signally contributed to this first volume, willing to act as associate editors for their assigned topic for the year. The responsibilities of the associate editor are by no means light: it is for him

to select, within the topic and the space assigned, the methods most worthy of description and the contributors best fitted to describe. Obviously the methods most suitable for description in this form are those which are of wide actual or potential application and which have not been published in full or have been usefully modified since publication; obviously, too, the inclusion of a method stamps it as being convenient and reliable in the associate editor's expert estimation, but it does not conversely follow that omitted methods are of lesser value. The associate editor may also send each contribution to another experienced investigator for comment and review.

As members of the Governing Board, we are very conscious of the lightness of our own responsibilities in comparison with those of the associate editors and, still more, those of our Dr. V. R. Potter, who, to our great satisfaction, agreed to assume the further ungrateful task of acting as Editor-in-Chief for the year, charged among other things with the duty of distributing space among the sections. Any values which this volume may have must be credited to the editors, the contributors and the referees, rather than to us. It remains for us merely to select topics and to try to find equally competent and conscientious editors for the next volume and its successors. To this end we should most gratefully receive and consider any suggestions that readers may care to send us.

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SECTION I

Assay of Antibiotics

ASSOCIATE EDITOR—*Henry Welch*

INTRODUCTION

THE DISCOVERY of penicillin by Sir Alexander Fleming and its rapid commercial development in this country stimulated widespread interest in antibiotics. Production of penicillin increased from a few million units in the latter months of 1942 to over 7000 billion units per month during 1948. The amazing production record is the result of the efforts of 15 drug manufacturers in this country who, during the early part of World War II, were responsible for supplying great quantities of this valuable therapeutic agent to the armed forces of this country and to those of our allies. The therapeutic evaluation of penicillin was planned and arranged by the Committee on Medical Research of the Office of Scientific Research and Development who, with the co-operation of scientists throughout the country, demonstrated the efficacy of this drug for the treatment of a great variety of diseases.

In the early studies of penicillin, biologic assays were utilized exclusively, but during the past three years, with the isolation of crystalline penicillin, both chemical and physical methods have been developed. These have proved to be quite satisfactory. The demonstration that penicillin as produced by the mold was not necessarily one substance but a combination of at least five penicillins, F (Δ^2 pentenyl penicillin), G (benzyl penicillin), X (P-hydroxybenzyl penicillin), K (n-heptyl penicillin) and dihydro F (n-amyl penicillin), has been of considerable value in the development of definitive methods.

Although amorphous penicillin, which was utilized in great quantities prior to March, 1946, had little or no toxicity, there has been a steady increase in the production of pure crystalline penicillin G since then, until now more than 90 per cent of the penicillin administered parenterally in clinical practice is crystalline material largely of the G type. There has been considerable reduction in the production of penicillin K

As compared to penicillin and streptomycin, bacitracin and tyrothricin are produced in relatively small amounts in this country. Because of their inherent toxicity, both are recommended for topical application only. There is a possibility that bacitracin, which is a polypeptide, may eventually be purified and utilized parenterally since conservative use of this drug systemically has already been successful in the hands of Meleney and his co-workers, the discoverers of this antibiotic. It is unlikely, however, that tyrothricin (a combination of 80 per cent tyrocidine and 20 per cent gramicidin), now produced in small amounts in crystalline form, will ever be satisfactory for parenteral use. The methods described here for both bacitracin and tyrothricin are tentative. In the case of the former substance, more accurate methods must await its eventual purification; tyrothricin preparations, on the other hand, frequently contain substances which have been found to interfere with the assay methods so far developed.

Test methods chosen for inclusion in this chapter obviously do not include all of the reliable methods to be found in the literature. The methods included have, in most cases, been given extensive trial and have proved satisfactory.

—Henry Welch.

which, although more active than penicillin G in vitro, has only a fraction of its activity in vivo. Control of the quantity of penicillin K is maintained by regulation, as is the control of all penicillin and streptomycin preparations, under an amendment to the Federal Food, Drug and Cosmetic Act. Penicillin for use in the body must not contain more than 30 per cent penicillin K, while products labeled as containing crystalline penicillin G must contain at least 85 per cent of this fraction by weight. Although no penicillin X is available on the market at this time, the regulations require a product to contain at least 90 per cent of a salt of penicillin X to be labeled as this fraction. Methods for the assay of penicillin for G, K and X content are included in this section.

Although it may be said that penicillin was discovered by accident, the discovery of streptomycin, our second most important antibiotic, by Waksman and his co-workers was the result of a carefully designed investigation. These workers were searching for an antibiotic antagonistic to gram-negative organisms to complement the activity of penicillin which is selective for gram-positive organisms. Unlike penicillin, streptomycin has some toxicity, particularly when it is used in 1–4 g daily doses for 1–3 months, as is done in the treatment of certain types of tuberculosis. Deafness and vertigo due to eighth nerve damage may result even when crystalline streptomycin (the calcium chloride, trihydrochloride double salt of streptomycin) is used for treatment. In the treatment of other diseases for which streptomycin is effective (*Hemophilus influenzae* meningitis, tularemia and gram-negative urinary tract infections) the total dosage is so low that few if any toxic reactions are observed.

The manufacturing experience gained by industry in this country in the development of penicillin production has been of great value in the development of production methods for streptomycin. As a result, in a relatively short time production of streptomycin reached 1,000,000 g per month early in 1947 and was double that by the end of the year. One manufacturer (the largest at this time) is now producing only crystalline material. As with penicillin, early indications were that streptomycin was a single entity, but it soon became evident that it consists of at least three different "streptomycins," streptomycin A, streptomycin B and a third fraction unclassified. These streptomycins differ in their activity against sensitive organisms and occur in commercial streptomycin in varying concentrations, depending on the manufacturer and the extraction procedures utilized. It can be said, however, that streptomycin A (which is approximately five times as active as streptomycin B) constitutes about 70 per cent of commercial amorphous streptomycin. The methods described for the assay of streptomycin in this section are both biologic and chemical. However, the fact that commercial streptomycin has been found to be not a single entity has delayed development of accurate chemical methods, although the methods used are quite sufficient for clinical evaluation of this drug.

As compared to penicillin and streptomycin, bacitracin and tyrothricin are produced in relatively small amounts in this country. Because of their inherent toxicity, both are recommended for topical application only. There is a possibility that bacitracin, which is a polypeptide, may eventually be purified and utilized parenterally since conservative use of this drug systemically has already been successful in the hands of Meleney and his co-workers, the discoverers of this antibiotic. It is unlikely, however, that tyrothricin (a combination of 80 per cent tyrocidine and 20 per cent gramicidin), now produced in small amounts in crystalline form, will ever be satisfactory for parenteral use. The methods described here for both bacitracin and tyrothricin are tentative. In the case of the former substance, more accurate methods must await its eventual purification; tyrothricin preparations, on the other hand, frequently contain substances which have been found to interfere with the assay methods so far developed.

Test methods chosen for inclusion in this chapter obviously do not include all of the reliable methods to be found in the literature. The methods included have, in most cases, been given extensive trial and have proved satisfactory.

—Henry Welch.

ASSAY OF PENICILLIN POTENCY: A. BIOLOGIC METHODS

1. COMMERCIAL PREPARATIONS

Cylinder-Plate Assay[1]

PROCEDURE

a) *Cylinders* (*cups*).—Use stainless steel cylinders with outside diameter 8 mm (±0.1 mm), inside diameter 6 mm (±0.1 mm) and length 10 mm (±0.1 mm).

b) *Culture media*.—Use ingredients that conform to standards prescribed by the U.S.P. or N.F.

1. Make nutrient agar for the seed layer and for carrying the test organism as follows:

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Glucose	1.0 g
Agar	15.0 g
Distilled water, q.s.	1000.0 ml
pH 6.5–6.6 after sterilization.	

2. Make nutrient agar for base layer as follows:

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Agar	15.0 g
Distilled water, q.s.	1000.0 ml
pH 6.5–6.6 after sterilization.	

3. Make nutrient broth for preparing an inoculum of the test organism as follows:

Peptone	5.0 g
Yeast extract	1.5 g
Beef extract	1.5 g
Sodium chloride	3.5 g
Glucose	1.0 g
Dipotassium phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Distilled water, q.s.	1000.0 ml
pH 7.0 after sterilization.	

[1] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

Instead of media prepared from the individual ingredients specified in paragraphs (b) (1), (2) and (3), media may be made from a dehydrated mixture which, when *reconstituted with distilled water*, has the same composition as such media. Minor modifications of the individual ingredients specified in paragraphs (b) (1), (2) and (3) are permissible if the resulting media possess growth-promoting properties at least equal to those of the media described.

c) *Working standard.*—Keep the working standard (obtained from the Food and Drug Administration) in tightly stoppered vials, which in turn are kept in larger stoppered tubes containing anhydrous calcium sulfate, constantly in the refrigerator at 15 C (59 F) or below. Weigh out carefully in an atmosphere of 50 per cent relative humidity or less between 4 and 5 mg of the working standard and dilute with sterile 1 per cent phosphate buffer (pH 6.0) to make a stock solution of any convenient concentration. Keep this solution at a temperature of about 10 C and use for 1 day only. From this stock solution make appropriate working dilutions.

d) *Preparation of sample.*—Dissolve aseptically, in sterile distilled water, the sample to be tested to make an appropriate stock solution.

e) *Preparation of plates.*—Add 21 ml of agar to each Petri dish (20 × 100 mm). Distribute the agar evenly in the plates and allow it to harden. Use the plates the same day they are prepared. The test organism is *Staphylococcus aureus* (FDA 209-P or American Type Culture Collection [ATCC] 9144). Maintain the test organism on agar slants and transfer to a fresh agar slant about once a week. Prepare an inoculum for the plates by transferring the culture from the agar slant into broth and incubate at 37 C. From 16 to 24 hr thereafter add 2.0 ml of this broth culture to each 100 ml of agar, which has been melted and cooled to 48 C. Mix the culture and agar thoroughly and add 4 ml to each of the plates containing the 21 ml of uninoculated agar. Tilt the plates back and forth to spread the inoculated agar evenly over the surface. Porcelain covers glazed on the outside are used. Place four cylinders on the agar surface so that they are at approximately 90° intervals on a 2.8 cm radius. In placing the cylinders drop them from a height of 1/2 in., using a mechanical guide or device.

A suspension of the test organism may be used in place of the broth culture in preparing the inoculum for the seeding of plates. Prepare such a suspension as follows: Wash the organisms from an agar slant, which has been incubated for 24 hr at 37 C and stored for 24 hr at room temperature, with 2.0 ml of sterile physiologic saline onto a large agar surface such as that provided by a Roux bottle containing 300 ml of agar. Spread the suspension of organisms over the entire agar surface with the aid of sterile glass beads. Incubate 24 hr at 37 C and store for 24 hr at room temperature. Wash the resulting growth from the agar surface with about 50 ml of sterile physiologic saline. Standardize this suspension by determining the dilution which will permit 20 per cent light transmission

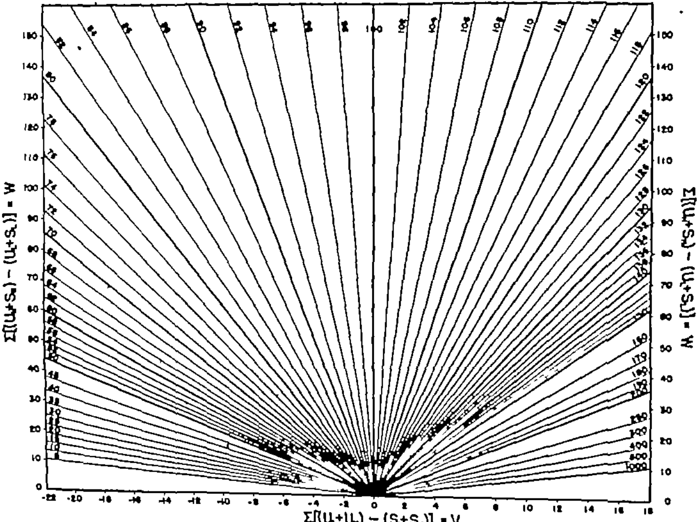

FIG. 1.—Penicillin assay. Chart for determining potency as percent of standard from two-dose four-plate method, ratio of doses 4:1. H = high dose; L = low dose; $H/L = 4$.

through a filter at 6500 A in a photoelectric colorimeter. Add 1.5–2.0 ml of this resulting dilution to each 100 ml of agar which has been melted and cooled to 48 C to prepare the inoculum for the plates. The suspension may be used for 1 week.

f) Assay.—Use four plates for each sample. Fill one cylinder on each plate with a 1.0 unit/ml dilution, and one with a 0.25 unit/ml dilution, of the working standard. Add the estimated dilutions of 1.0 unit/ml and 0.25 unit/ml of the sample under test to the remaining two cylinders on each plate. Carefully place the plates in racks and incubate 16–18 hr at 37 C. After incubation, measure the diameter of each circle of inhibition to the nearest 0.5 mm, using a colony counter with a millimeter scale etched into the supporting glass over the light source. Other measuring devices of equal accuracy may be used.

g) Estimation of potency and error.—1. Use the chart (Fig. 1) and nomograph (Fig. 2) for estimating potency and its standard error. To use the chart for estimating potency two values, namely, V and W, are required. For each plate calculate two values.

$$v = (U_L + U_H) - (S_L + S_H)$$

and

$$w = (U_H + S_H) - (U_L + S_L),$$

where S_H and S_L are the diameters of the zones of inhibition in millimeters of the 1.0 unit and 0.25 unit dilutions of the standard, respectively, and U_H and U_L refer similarly to the corresponding dilutions of the sample under test. The value of V is the sum of the v values for all plates and W is the sum of the w values for all plates. To estimate potency, locate the point on the chart corresponding to the values of V and W; the potency can be read from the radial lines on the chart.

2. The standard error of the assay is estimated by using the nomograph (Fig. 2) which requires five values, namely, the potency, V, W, Rv and Rw. Rv (range of the v's) is the highest value of v minus the lowest value of v obtained from the individual plates. Similarly, Rw is the difference between the highest and the lowest w value. After obtaining these five values, connect with a straightedge the points corresponding to v and w on the respective scales on the right nomograph (Fig. 2*B*). Mark with a pin or sharp-pointed pencil the intersection of the straightedge and the diagonal line of the nomograph. Move the straightedge so that it connects the value of Rw on its scale and the diagonal line at the point of the pin. The value for Q is thus determined by the scale value where the straightedge crosses the line labeled "Q." T is obtained by adding the squares of Q and Rv. On the left nomograph (Fig. 2*A*) connect the values of T and W with the straightedge and read the value of the ratio (standard error of assay–potency) where the straightedge intersects the scale of values for the ratio. This value multiplied by the potency equals the percentage standard error of the assay. The standard error of the assay calculated here estimates only how closely one assayist can check himself on

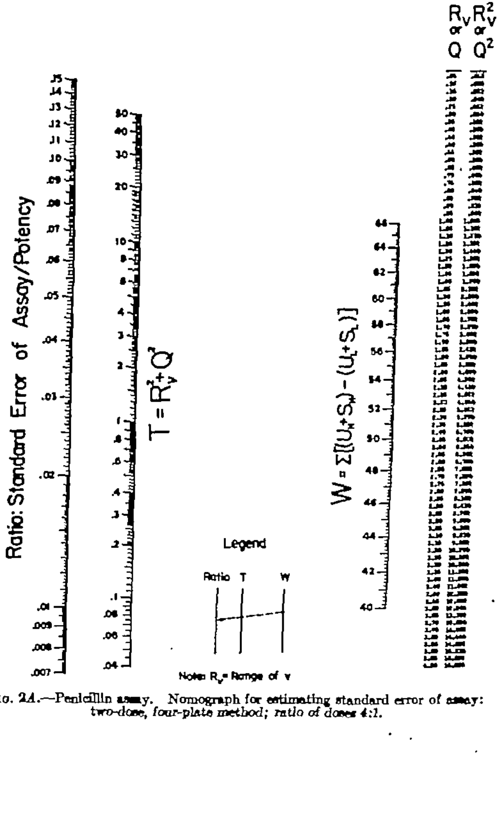

FIG. 24.—Penicillin assay. Nomograph for estimating standard error of assay: two-dose, *four-plate method; ratio of doses 4:1.*

any given set of dilutions of unknown and standard. It does not include any errors of weighing or errors due to variations in materials or subdivisions of a lot of penicillin.

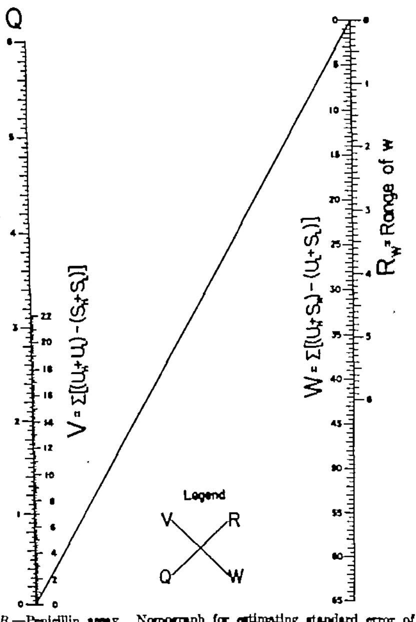

FIG. 2B.—Penicillin assay. Nomograph for estimating standard error of assay: two-dose, four-plate method; ratio of doses 4:1.

Figure 1 should not be used for determinations of potency lower than 50 per cent or higher than 150 per cent of the standard. If potency lies outside these limits, the assay should be repeated with a higher or lower dilution. The radial lines on the chart beyond these limits permit rough estimation of potency from as low as 5 per cent to as high as 1000 per

cent when low values of W are found. If the value of V or W falls outside the limits of the chart, divide both V and W by the same proper number to bring them into the range of the chart and read the potency from the radial lines as before. If 11.4 Rw is greater than W, the slope of the assay does not differ significantly from zero and the assay is invalid. (The figure 11.4 was obtained by use of Student's "t" test for determining the significance of a slope.)

In certain laboratories it has been noted that with the 4:1 ratio, involving concentrations of 0.25 unit for the low dose, the zone of inhibition given by this dose may either be too small for accurate reading or have edges which are poorly defined. To permit use of a higher concentration of penicillin for the low dose, a chart (Fig. 3) may be used in assays in which the ratio of doses is 2:1, i.e., the high dose (sH) is twice the low dose (sL). As in Figure 1, if the potency lies outside the limits of 50 and 150 per cent the assay should be repeated with a lower or higher dilution. The potencies beyond these limits are to be used for rough estimation purposes only. These extensions can also be used for four (or more) plate assays if both V and W are divided by the same proper number to bring them into the range of the chart. The standard error of the assay using the ratio of doses of 2:1 is estimated by employing the nomograph (Fig. 2) in the same manner as described for the 4:1 ratio of doses. However, the resultant standard error of the assay derived in this manner must be divided by 2 to give the correct standard error of the assay for the 2:1 ratio of doses.

Potency of a sample may also be determined by the *standard curve technique*, using a single dose of standard and unknown.

Dilute the sample to be tested to 1.0 unit/ml (estimated) in 1 per cent phosphate buffer pH 6.0. Place six cylinders on the inoculated agar surface at approximately 60° intervals on a 2.8 cm radius. Use three plates for each sample. Fill three cylinders on each plate with the 1.0 unit/ml standard and three cylinders with the 1.0 unit/ml (estimated) sample, alternating standard and sample. Incubate the plates for 16–18 hr at 37 C and measure the diameter of each circle of inhibition. At the same time prepare a standard curve using concentrations of the standard of 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4 and 1.5 unit/ml in sterile 1 per cent phosphate buffer pH 6.0. Use three plates for determination of each point on the curve, a total of 27 plates. On each of three plates fill three cylinders with the 1.0 unit/ml standard and the other three cylinders with the concentration under test. Thus there will be 81 one-unit determinations and nine determinations for each of the other points on the curve.

After the plates have incubated, read the diameters of the circles of inhibition. Average the readings of the 1.0 unit/ml concentrations and the readings of the point tested for each set of three plates, and average also all 81 readings of the 1.0 unit/ml concentration. The average of the 81 readings of the 1.0 unit/ml concentration is the correction point for the curve. Correct the average value obtained for each point to the figure it

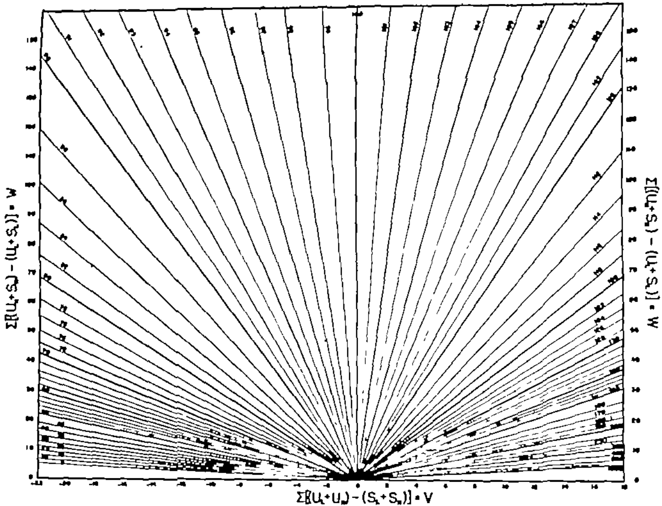

FIG. 3.—Penicillin assay. Chart for determining potency as percent of standard from two-dose four-plate method, ratio of doses 2:1. H=high dose; L=low dose; H/L=2.

would be if the 1.0 unit/ml reading for that set of three plates were the same as the correction point. Thus, if in correcting the 0.8 unit/ml concentrations the average of the 81 readings of the 1.0 unit/ml concentration is 20.0 mm, and the average of the 1.0 unit/ml concentration of this set of three plates is 19.8 mm, the correction is 0.2 mm. If the average reading of the 0.8 unit/ml concentration of these same three plates is 19.0 mm, the corrected value is then 19.2 mm. Plot these corrected values, including the average of the 1.0 unit/ml concentration, on 2 cycle semilog paper using the concentration in units/ml as the ordinate (the logarithmic scale) and the diameter of the zone of inhibition as the abscissa. Draw the standard curve through these points. The 10 points selected to determine the curve are arbitrary and should be so chosen that the limits of the curve will fill the needs of the laboratory. However, the potency of the sample under test should fall in the interval of from 60 to 150 per cent of the correction point of the standard curve.

To estimate potency of the sample, average the zone readings of the standard and the zone readings of the sample on the three plates used. If the sample gives a larger average zone size than the average of the standard, add the difference between them to the 1.0 unit/ml zone on the standard curve. If the average sample value is lower than the standard value, subtract the difference between them from the 1.0/ml unit value on the curve. From the curve read the potencies corresponding to these corrected values of zone sizes.

Filter Paper Disk Method[3]

PROCEDURE

Transfer the test organism, *Staphylococcus aureus* (strain H), from an agar slant through peptone broth for two 24 hr growth periods at 37 C. Hold the second transfer at 5 C for 16–18 hr.

Pipet 10 ml of fresh nutrient agar into uniform, flat-bottomed Petri dishes and incubate for 16–18 hr at 37 C. Hold these plates in the refrigerator for at least 1 hr.

Flood each plate with 1 ml of refrigerated culture. Remove the excess with a capillary pipet, dry the plates for 1 hr at 37 C (use wooden racks which support the top half of the Petri dish above the bottom half so that there is about 1/2 in. clearance). Store the plates in the refrigerator, inverted, at least 1 hr.

Sterilize filter paper disks by dry heat and immerse in the test fluid until saturated (30 sec). Remove from the fluid with sterile forceps and gently shake off the excess, place on the seeded plates and set the disks in place with special care without smearing the surface of the agar. Place three filter papers, evenly spaced, in each plate. Run triplicate plates for

[3] Vincent, J. G., and Vincent, H. W.: Filter paper disc modification of the Oxford cup penicillin determinations, Proc. Soc. Exper. Biol. & Med. 55: 162, 1944.

greater accuracy. One of the disks on each plate may be a standard penicillin control.

Incubate the plates, not inverted, at 37 C for 14 hr (placed on a wooden block to avoid excess condensation).

A clear well defined zone of inhibition will appear around each disk when viewed with a diffuse light if the test fluid contains more than $^1/_{16}$ unit of penicillin. Compare the diameters of the zones with a standard curve of a known sample to determine the concentration of penicillin.

NOTES

1. Dilute the sample to approximately 1 unit/ml for best results. Penicillin samples containing more than 4 units/ml can be measured with the disk method, but with no great accuracy.

2. Nutrient broth, 1 per cent peptone, 0.5 per cent NaCl.

3. Filter paper disks no. 740-E, $^1/_2$ in. diameter. Schleicher and Schuell Company, Inc., New York.

Rapid Determination of Susceptibility to Penicillin and Streptomycin[3]

PROCEDURE

Cut disks, 0.5 mm in diameter, from Whatman no. 2 filter paper. Sterilize in a hot air oven. Prepare solutions of penicillin, 15 units/ml, and of streptomycin, 500 μg/ml. Streak blood plate(s) with clinical specimen(s). Dip one of the disks into the solution of penicillin with alcohol-flamed, fine-pointed forceps. Remove excess solution by placing the disk flat against the wall of the tube. Place the saturated disk on the left side of the inoculated plate in the area of the primary inoculum. Place the disk saturated with the solution of streptomycin on the right side of the plate. After overnight incubation examine the plates for predominating organisms. Measure zones of inhibition around the disks with a millimeter ruler. Evaluate relative susceptibility of organisms according to zone size as in the tabulation.

Agent	Zone of Inhibition, Mm.	Suscept. Range	Rel. Suscept.
Penicillin	>20	<0 1 unit/ml	Very
	10–20	0 1–0.4 unit/ml	Mod.
	<10	>0.4 unit/ml	Resist.
Streptomycin	>15	<4 μg/ml	Very
	10–15	4–25 μg/ml	Mod.
	<10	>25 μg/ml	Resist.

NOTES

1. Standardized solutions of the antibiotic should be stored in the refrigerator. Fresh solutions should be prepared at frequent intervals.

[3] Bondi, A.; Spaulding, E. H.; Smith, D. E., and Dietz, C. C.: Routine method for rapid determination of susceptibility to penicillin and other antibiotics, Am. J. M. Sc. 214: 221, February, 1947.

Solutions are best prepared in N/15 phosphate buffer, pH 6 for penicillin and pH 8 for streptomycin.

2. Both disks should be placed in comparable positions with respect to the inoculum. The amount of growth influences zone size, although slight variations in zone size do not materially influence final evaluation of relative susceptibility.

3. Heavy growth of resistant organisms may mask zones of inhibition of susceptible organisms. In such instances it may be necessary to subculture to fresh plates and repeat the tests.

4. Since activity of streptomycin is reduced under anaerobic conditions, only aerobic plates should be used in evaluating susceptibility to this agent. This is not true of penicillin.

5. The method may be used for testing susceptibility to any other antibacterial agent which is water-soluble and diffusible.

6. The rather broad limits of susceptibility by this method are sufficiently accurate to serve as a guide in therapy.

Types of Penicillin in Mixtures[4]

PROCEDURE

For each of the three assay organisms (see Notes) prepare 50 tubes (18 × 150), each containing 10 ml of sterile medium (see Notes). Prepare sterile solutions of standards for each type of penicillin at two concentration levels (10 and 2.5 units/ml) and add them aseptically in graded levels with a micropipet to cover the ranges indicated in the tabulation.

PENICILLIN INHIBITION RANGES IN UNITS/ML

Organisms	Type of Penicillin			
	G	X	F	K
Staph. aureus	0.0 –0.025	same as for penicillin G		
B. brevis	0.01 –0.03	0.02 –0.06	0.03–0.07	0.05–0.12
Organism E	0.015–0.03	0.0075–0.02	0.03–0.06	0.04–0.10

Use about 10 tubes per penicillin type per organism. Add the unknown in graded levels as for the standards. Inoculate with 1 drop per tube of a 15 hr culture of the appropriate organism for each set. (Inocula are grown in the regular assay media; their turbidity measured in per cent transmission should be about 65 for *Staph. aureus*, 30 for *B. brevis* and 60 for organism E.) Incubate *Staph. aureus* tubes for 15 hr at 37 C, *B. brevis* tubes for 15 hr at 37 C on a shaker and organism E for 15 hr at 45 C in a water bath. Read turbidity on an Evelyn photoelectric colorimeter with a 660 μ filter. Plot galvanometer readings against units/ml. (The last values are determined from the *Staph. aureus* curve obtained with crys-

[4] Higuchi, K., and Peterson, W. H.: Estimation of types of penicillin in mixtures by differential microbiological assay, Ind. Eng. Chem., anal. ed. 19: 68, 1947.

talline penicillin G of 1667 units/mg potency.) For a level of inhibition with the unknown selected about in the middle of the growth curves, calculate the ratio of potency of penicillin X, F or K as compared to G and use these values in the equations illustrated below. A typical example for a mixture containing penicillin G, X and K was found to calculate as follows:

Calculated for 100 units of the unknown:

$G + X + K = 100$ (*aureus* assay value)
$G + 0.55X + 0.20K =$ ——— (*brevis* assay value in terms of G)
$G + 1.80X + 0.31K =$ ——— (organism E assay value in terms of G)

The letters represent the amount in per cent of the designated penicillin that is present. Solve the set of simultaneous equations for composition of the mixture. Since the constants representing the ratios of penicillin potencies as compared to G vary somewhat, they must be determined at each assay.

NOTES

1. The three organisms are *Staph. aureus* 209-P, *B. brevis* ATCC 8185 and an unidentified spore-forming lactic organism designated as E. All are carried on 0.2 per cent glucose, 0.3 per cent Difco yeast extract agar. The method is strictly applicable only when the types of penicillin present are known and no more than three are present. However, the data are of value even under more complex conditions.

2. Composition of assay media (g/liter): *Staph. aureus* medium: Difco Yeast Extract 3.0, Bacto Peptone 6.0, glucose 2.0, Armour Beef Extract 1.5. *B. brevis* medium: Difco Yeast Extract 3.0; Bacto Peptone 6.0. Organism E medium: Difco Yeast Extract 3.0, Bacto Peptone 6.0, glucose 2.0, KH_2PO_4 5.0, K_2HPO_4 0.5.

Differential Assay Procedures

I. Cylinder-Plate Technique—*Staphylococcus aureus* FDA 209-P test organism

PROCEDURE

Place 80 ml of sterile base agar (medium I, p. 10) in the sterile Peoria type assay tray and allow to harden at room temperature. Drop stainless steel cylinders on the hard agar, then transfer the trays to the 55 C incubator preparatory to flooding with 48 C flooding agar (medium II) containing the inoculum.

Prepare a standard solution of penicillin G containing 1 unit/ml in 1 per cent phosphate buffer pH 6.0. One or more dilutions of the samples are prepared in buffer to contain an estimated 1 unit/ml. One third of the cylinders on the assay tray are devoted to the standard and two

[a] Schmidt, W. H., Schenley Laboratories, Lawrenceburg, Ind. Unpublished data.

thirds to the samples. Fill the cylinders level with the top with the penicillin solutions. Incubate the trays at 37 C for approximately 15 hr. Measure and record the zones of inhibition to the nearest 0.1 or 0.2 mm. Calculate potency by means of a standard curve prepared by plotting the average value of 100 determinations on each of five different concentrations of penicillin ranging from 0.25 to 2.5 units/ml. Plot the curve either as concentration vs. diameter of zone of inhibition or log of concentration vs. diameter squared. With this curve convert zones of inhibition of unknowns to units/ml and multiply by the dilution factor.

NOTES

1. Petri dishes may be used in place of the Peoria type assay tray.
2. Maintain stock cultures of *Staph. aureus* FDA 209-P on tryptose agar (medium III). Store slants in refrigerator after growth has occurred.
3. The cell suspension used for inoculating the flooding agar is prepared as follows:

a) Transfer inoculum from a slant culture to a flask containing 50 ml of brain heart infusion broth (medium IV). Incubate at 37 C for 16 hr.

b) Streak from broth one tryptose agar plate (medium III) and incubate 16 hr at 37 C.

c) Pick well isolated deep orange colonies, streak three tryptose agar slants and incubate for 16 hr at 37 C.

d) Wash cells from slants with sterile 1 per cent buffer pH 6.0 and use the suspension to inoculate a bottle containing 300 ml of tryptose agar (medium III). Draw off excess fluid after the surface of the agar has been completely covered. Incubate for 16 hr at 37 C and allow the culture to stand at room temperature for another 24 hr before removing the cells.

e) Wash cells off the agar in the bottle with 25 ml of sterile 1 per cent phosphate buffer pH 6.0. Store in a sterile bottle in the refrigerator.

II. Cylinder-Plate Technique—*Bacillus subtilis* NRRL B-558RB test organism

PROCEDURE

The procedure is the same as that for *Staph. aureus* except:

1. Substitute medium V for medium II.

NOTES

1. Maintain the organism on medium VI. Plates are streaked daily on this medium and rough colonies are selected the following day for transfer to broth. Incubate the plates at 30 C.
2. Medium VII is inoculated with rough type colony, then shaken for 15 hr at 30 C. Use 2 ml of this broth culture per 100 ml of flooding agar.

III. Cylinder-Plate Technique—*Bacillus subtilis* NRRL B-558S test organism

PROCEDURE

The procedure is the same as that for *Staph. aureus* except:

1. Substitute medium VIII for medium II.
2. Incubate trays at 30 C.

NOTES

1. Maintain the organism on medium VIII. After 15 hr growth at 30 C, place in the refrigerator. Replace the stock culture every four days.

2. Inoculum is produced by growing the organisms in medium IX with shaking for 15 hr at 30 C. Use 1 ml of broth culture per 100 ml of flooding agar.

IV. Cylinder-Plate Technique—*Leuconostoc dextranicum* NRRL B-640 test organism

PROCEDURE

The procedure is the same as that for *Staph. aureus* except:

1. Substitute medium X for medium II.
2. Incubate trays at 25–28 C.

NOTES

1. The cell suspension used for inoculating the flooding agar is prepared as follows:

a) Wash cells from one or two slants (medium III) with 8–10 ml of 1 per cent sterile buffer pH 6.0.

b) Inoculate a bottle containing 300 ml of medium X. Incubate at 25–28 C for 16 hr. At the same time inoculate one flask of B-640 broth (medium XI) to be incubated at room temperature and shaken on a Burrell shaker for 16 hr.

c) Wash cells from the agar surface with 25 ml of sterile buffer pH 6.0 and place the suspension in the refrigerator. Approximately 0.2 per cent inoculum (0.2 ml of cell suspension per 100 ml of flooding agar) is used for seeding the flooding agar (medium X). The cell suspension must be renewed weekly.

d) Use the broth culture to prepare additional slants.

2. The assay trays must be used shortly after the flooding agar has hardened or the trays must be refrigerated. This precaution is necessary because the organism grows rapidly at usual room temperatures.

GENERAL NOTES

Penicillin K and X analyses.—The relative responses of the different test organisms to the various penicillins follow.

TYPE OF PENICILLIN	209P	558RB	558S	610
G	100	100	100	100
F	100	05	05	75
K	100	30	30	50
X	100	120–100	00–120	175–225

When more than two types of penicillin are present in a sample the differential assay loses its unique value. However, separation of the penicillins by extraction is exceedingly helpful in obtaining reliable data. The following extraction procedures provide means for preparing samples and for calculating results.

I. Penicillin K analysis
  A. Preparation of sample
    1. Adjust sample to pH 6.0 with 0.3M H_3PO_4 or 0.3M NaOH.
    2. Extract with 2 volumes of freshly washed $CHCl_3$.
    3. Extract twice from $CHCl_3$ with 0.3M phosphate buffer at pH 7.5.
    4. Submit for assay
      a) Initial sample,
      b) Extracted portions,
      c) Residual aqueous portion.
  B. Assay procedure. All samples are assayed against *Staph. aureus.* In addition, the extracted portion is assayed against either strain of *B. subtilis,* B-558RB or B-558S.

    Prepare a chart plotting percentage K on the X axis and assay ratios, *B. subtilis–Staph. aureus* on the Y axis. A line is drawn connecting the point 100 per cent K on the X axis with 0.05 on the Y axis. Plot the assay ratio obtained by *B. subtilis–Staph. aureus* assay of the extracted material on this line and read the percentage K from the graph. Multiply this value by 8 to give total units of K in the original sample.

II. Penicillin X analysis
  A. Preparation of sample
    1. Extract sample with $CHCl_3$ at pH 2.0, maintaining system at 2 C.
    2. Extract $CHCl_3$ twice with phosphate buffer at pH 7.5.
    3. Submit for assay
      a) Aqueous residue of original sample,
      b) Extracted portion.
  B. Assay procedure. Assay all samples on *Staph. aureus* and on *L. dextranicum; B. subtilis* B-558RB may be used in lieu of *L. dextranicum,* but the latter is preferred. Prepare a chart plotting percentage X on the X axis and assay ratios *L. dextranicum–Staph. aureus* on the Y axis. The assay ratio obtained by differential assay of the extracted material becomes a point on the Y axis representing 0 per cent X. The assay ratio obtained by differential assay of a pure X standard becomes a point on the

Y axis representing 100 per cent X. Connect these two points. Plot the ratio of the residual material on this line and read percentage X in residual material from the graph.

Media

Medium I (base agar)

N Z Case*	10.0 g
Beef Extract (Difco)	1.5 g
Yeast Extract (Difco)	3.0 g
Agar	17.0 g
Distilled water, q.s.	1000.0 ml

Medium II (flooding agar for *Staph. aureus*)

N Z Case*	10.0 g
Beef Extract (Difco)	1.5 g
Yeast Extract (Difco)	3.0 g
Dextrose	1.0 g
Agar	17.0 g
Orange vegetable dye (optional)	0.5 g
Distilled water, q.s.	1000.0 ml

Medium III (stock culture medium for *Staph. aureus*)

Tryptose (Difco)	20.0 g
Dextrose	1.0 g
Sodium chloride	5.0 g
Agar	20.0 g
Distilled water, q.s.	1000.0 ml

Medium IV (broth culture medium for *Staph. aureus*)

Brain Heart Infusion Broth (Difco)	37.0 g
Distilled water, q.s.	1000.0 ml

Medium V (flooding agar for *B. subtilis* NRRL B-558RB)

Peptone (Difco)	5.0 g
Casitone† or N Z Case	5.0 g
Yeast Extract (Difco)	3.0 g
Beef Extract (Difco)	1.5 g
Glucose	10.0 g
Agar	16.0 g
Blue vegetable dye (optional)	0.2 g
Distilled water, q.s.	1000.0 ml

Medium VI (for maintaining *B. subtilis* NRRL B-558RB)

Peptone (Difco)	5.0 g
Casitone (Difco)	5.0 g
Yeast Extract (Difco)	3.0 g
Agar	20.0 g
Distilled water, q.s.	1000.0 ml

Medium VII (for broth culture of *B. subtilis* NRRL B-558RB)
Same as medium VI except for elimination of agar

Medium VIII (flooding agar for *B. subtilis* NRRL B-558S)

Peptone (Difco)	10.0 g
Yeast Extract (Difco)	3.0 g
Glucose	8.0 g
Agar	16.0 g
Red vegetable dye (optional)	0.2 g
Distilled water, q.s.	1000.0 ml

* A casein hydrolysate made by Sheffield Farms, Inc.
† A casein hydrolysate made by Difco.

Medium IX (broth medium for *B. subtilis* NRRL B-558S)

Peptone (Difco)	5.0 g
Yeast Extract (Difco)	3.0 g
Beef Extract (Difco)	1.5 g
Glucose	8.0 g
K_2HPO_4	2.5 g
KH_2PO_4	2.5 g
Distilled water, q.s.	1000.0 ml

Medium X (flooding and inoculating agar for *L. dextranicum* NRRL B-640)

N Z Case	12.0 g
Yeast Extract (Difco)	5.0 g
Beef Extract (Difco)	3.0 g
Yellow vegetable dye (optional)	0.5 g
Agar	16.0 g
Distilled water, q.s.	1000.0 ml

Medium XI (broth medium for *L. dextranicum* NRRL B-640)
Same as medium X except for elimination of the dye and the agar

Three-Hour Cylinder-Plate Assay[a]

PROCEDURE

a) Preparation and incubation of plates.

1. Prepare and seed plates with *Staph. aureus* as for the standard cup-plate method of assay (p. 5).

2. Incubate seeded plates for 3 hr at 38 C. This is the pre-incubation period without penicillin.

3. Store plates in a refrigerator for a few hours or days until needed. Pre-incubated plates have been used with entirely satisfactory results after 5 days in a refrigerator.

4. Place standard penicylinders and fill with solutions to be tested after the pre-incubated plates have come to room temperature.

5. Re-incubate plates for 3 hr at 38 C (postincubation). Satisfactory images may be developed on plates with 2.5 hr of postincubation under favorable conditions, but for routine assays, plates with 3 hr of post-incubation are preferred because the boundaries between zones of inhibition and noninhibition are sharper.

b) Physical development.—After removal from the incubator, treat the plates as follows:

1. Flood with about 30 ml of a 0.1 per cent aqueous solution of silver nitrate without removing cylinders.

2. Expose for 2.5–3 min to illumination from two 40 w daylight fluorescent lamps mounted in a reflector* at a distance of about 35 cm. This is equivalent to approximately 350 ft-c. Additional short exposures to ordinary laboratory illuminations are not detrimental.

3. Remove excess silver nitrate solution with a suction device.

[a] Goyan, F. M.; Dufrenoy, J.; Strait, L. A., and Pratt, R.: Three-hour "physical development" cup-plate assay for penicillin, J. Am. Pharm. A., sc. ed. 36: 65, 1947.
*General Electric Company Catalogue no. 59G283.

4. Add 30 ml of physical developer and allow it to remain for 7–10 min at room temperature.

5. Measure the diameters of the zones of inhibition in the conventional way.

Directions for preparation and use of developer:

Stock solution A

1. Dissolve 80 g of sodium thiosulfate and 20 g of anhydrous sodium sulfite in 300 ml of distilled water.
2. Dissolve 8 g of silver nitrate in 200 ml of distilled water.

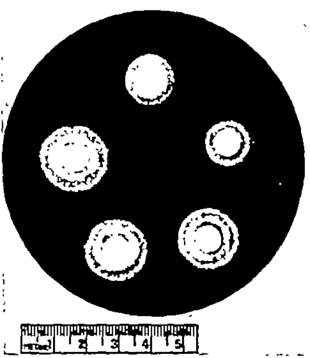

FIG. 4.—Typical penicillin assay plate processed by impregnation with silver and physical development after 3 hr of secondary incubation and diffusion of penicillin. Zones for solutions containing 1, 0.5, 2, 4 and 8 units/ml, respectively, are arranged in clockwise order beginning at the top.

3. Slowly pour solution 2 into solution 1 while stirring. The order of mixing and the stirring are important.

Stock solution B

1. Dissolve 20 g of sodium sulfite in 300 ml of distilled water, and when solution is completed add 3.8 g of 2,4-diaminophenol hydrochloride (Eastman Kodak Co. no. P 614) and stir until dissolved.

Just before use, mix equal parts of solutions A and B and dilute their combined volume with 3 volumes of distilled water. We have found this dilution best for our use, but the optimal concentration to be employed for varying conditions of light intensity, temperature of development,

Medium IX (broth medium for *B. subtilis* NRRL B-558S)

Peptone (Difco)	5.0 g
Yeast Extract (Difco)	3.0 g
Beef Extract (Difco)	1.5 g
Glucose	8.0 g
K_2HPO_4	2.5 g
KH_2PO_4	2.5 g
Distilled water, q.s.	1000.0 ml

Medium X (flooding and inoculating agar for *L. dextranicum* NRRL B-640)

N Z Case	12.0 g
Yeast Extract (Difco)	5.0 g
Beef Extract (Difco)	3.0 g
Yellow vegetable dye (optional)	0.5 g
Agar	16.0 g
Distilled water, q.s.	1000.0 ml

Medium XI (broth medium for *L. dextranicum* NRRL B-640)
Same as medium X except for elimination of the dye and the agar

Three-Hour Cylinder-Plate Assay[4]

PROCEDURE

a) *Preparation and incubation of plates.*

1. Prepare and seed plates with *Staph. aureus* as for the standard cup-plate method of assay (p. 5).

2. Incubate seeded plates for 3 hr at 38 C. This is the pre-incubation period without penicillin.

3. Store plates in a refrigerator for a few hours or days until needed. Pre-incubated plates have been used with entirely satisfactory results after 5 days in a refrigerator.

4. Place standard penicylinders and fill with solutions to be tested after the pre-incubated plates have come to room temperature.

5. Re-incubate plates for 3 hr at 38 C (postincubation). Satisfactory images may be developed on plates with 2.5 hr of postincubation under favorable conditions, but for routine assays, plates with 3 hr of postincubation are preferred because the boundaries between zones of inhibition and noninhibition are sharper.

b) *Physical development.*—After removal from the incubator, treat the plates as follows:

1. Flood with about 30 ml of a 0.1 per cent aqueous solution of silver nitrate without removing cylinders.

2. Expose for 2.5–3 min to illumination from two 40 w daylight fluorescent lamps mounted in a reflector* at a distance of about 35 cm. This is equivalent to approximately 350 ft-c. Additional short exposures to ordinary laboratory illuminations are not detrimental.

3. Remove excess silver nitrate solution with a suction device.

[4] Goyan, F. M.; Dufrenoy, J.; Strait, L. A., and Pratt, R.: Three-hour "physical development" cup-plate assay for penicillin, J. Am. Pharm. A., sc. ed. 36: 65, 1947.
*General Electric Company Catalogue no. 59G283.

4. Add 30 ml of physical developer and allow it to remain for 7–10 min at room temperature.

5. Measure the diameters of the zones of inhibition in the conventional way.

Directions for preparation and use of developer:

Stock solution A

1. Dissolve 80 g of sodium thiosulfate and 20 g of anhydrous sodium sulfite in 300 ml of distilled water.
2. Dissolve 8 g of silver nitrate in 200 ml of distilled water.

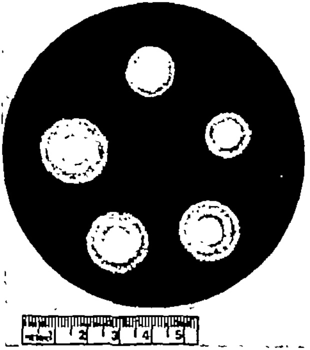

FIG. 4.—Typical penicillin assay plate processed by impregnation with silver and physical development after 3 hr of secondary incubation and diffusion of penicillin. Zones for solutions containing 1, 0.5, 2, 4 and 8 units/ml, respectively, are arranged in clockwise order beginning at the top.

3. Slowly pour solution 2 into solution 1 while stirring. The order of mixing and the stirring are important.

Stock solution B

1. Dissolve 20 g of sodium sulfite in 300 ml of distilled water, and when solution is completed add 3.8 g of 2,4-diaminophenol hydrochloride (Eastman Kodak Co. no. P 614) and stir until dissolved.

Just before use, mix equal parts of solutions A and B and dilute their combined volume with 3 volumes of distilled water. We have found this dilution best for our use, but the optimal concentration to be employed for varying conditions of light intensity, temperature of development,

etc., should be determined empirically in each laboratory. Stock solutions A and B should be stored in a refrigerator.

NOTE

The physical development method has been applied to plates seeded with various test organisms (notably *B. subtilis*, *B. coli*, etc.), preincubated and submitted to secondary incubation to allow diffusion of penicillin or of various other antibiotics (notably streptomycin); the respective optimal durations of pre- and secondary incubations vary with the test organism and the antibiotic.

Turbidimetric Assay[7]

Culture and inoculum.—Carry stock cultures of *Staph. aureus* (strain H) on plain nutrient agar slants. Incubate duplicate tubes of the organism at 37 C for 24 hr, then hold in the refrigerator. Use one culture for preparing inocula from day to day. Prepare two more slants at the end of the week, and at weekly intervals thereafter, from the unused culture. Check the new cultures by a Gram stain to see whether they are pure, discard the old cultures and use the new cultures as before.

Make the inoculum for the test by transferring a bit of the surface growth from the slant to one or more flasks containing 100 ml of plain nutrient broth (8 g of Difco Bacto Dehydrated Powder/l of water). Incubate the inoculated medium until the next day and use in the test. Incubation time is usually 14–18 hr.

Basal medium.—Composition per liter: Difco Bacto Nutrient Broth 16 g, yeast extract 4 g. Place varying amounts of the basal medium (depending on the number of tubes being used per test) in flasks, stopper with cotton plugs and autoclave at 15 lb for 15–20 min. Cool the flasks and put away for use as needed.

Buffer.—Make a stock buffer containing 10 g of KH_2PO_4 per liter and adjust to pH 6.8 with KOH or NaOH. Dilute this 1:10, autoclave and use to dilute standards and samples.

Standard solution.—Dilute a weighed portion of crystalline sodium penicillin G (potency 1667 units/mg) in volumetric flasks so that a solution containing 5 units/mg is obtained.

Samples.—If the samples are heavily contaminated, filter through a Seitz filter; otherwise merely dilute to proper concentration with sterile buffer. Ordinarily, the approximate potency of the material to be tested is known, and a final solution for the test may be prepared that will have a potency of 2–5 units/ml.

Equipment.—Set the test up in stainless steel racks holding forty $^1/_2 \times 4$ Kimble glass tubes covered with an inverted stainless steel tray $^1/_2$ in. deep to prevent dust contamination. Pipet standards and samples

[7] McMahan, J. R.: Improved short time turbidimetric assay for penicillin. J. Biol. Chem. 153:249, April, 1944.

with a special pipet prepared from a standard 0.2 ml Pyrex pipet.* If micropipets are used the small volume employed (0–0.16 ml) makes an insignificant difference in the final volume of medium in the tube (7 ml) and hence no buffer need be added to bring all the samples to a definite volume. If it is desired, a fixed sample volume is selected at say 1 or 2 ml so that 1 or 2 ml pipets may be used. If this is done, all samples added must be made up to the selected volume by addition of buffer solution and appropriate increase made in the concentration of the basal medium.

PROCEDURE

Place the test tubes in racks and cover with the inverted trays and, with the pipets, dry-sterilize at 170 C for $1^1/_2$ hr, cool and keep in reserve for use as needed. Establish the standard curve by pipetting the following amounts of standard solution (containing 5.0 units/ml) into a series of 12 test tubes: 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0, 0, 0.08, 0.06, 0.04, 0.02 ml. These points correspond to values of 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 and 0 unit.

Pipet in another series of tubes, volumes of the penicillin solutions to be tested: 0.16, 0.14, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.08, 0.06, 0.04, 0.02 ml. As previously stated, the primary dilutions of the unknown solutions are made up so that their concentration falls in the range of 2–5 units/ml. Inoculate a flask containing sufficient basal medium to fill all the tubes in the test with the 14–18 hr inoculum. Use 65 ml of inoculum per liter of basal solution. Use a Brewer automatic pipetting machine to add the inoculated basal medium to the tubes. Keep it filled with 80 per cent alcohol when not in use and rinse out by pumping sterile water through it just before use. Put the suction and delivery tubes into the flask and recirculate the inoculated medium until all bubbles are out of the delivery system and the medium is well mixed. Put one 7 ml portion of medium in each tube in the test, cover the tubes with the inverted trays and place the racks in a mechanically stirred water bath incubator at 37 C ($\pm 0.1°$). Leave the racks in the bath for $3^1/_4$ hr. Incubation times of $3^1/_2$–4 hr may be used. Remove the racks at the end of the incubation time and steam 10 min in an Arnold sterilizer or in an autoclave with the steam escape valve open. The effect of steaming is twofold: (1) it kills the cells and thus prevents additional growth during the reading of the test, and (2) the organisms are no longer dangerous to handle in the reading process. Remove the racks from the sterilizer and put in a water bath to cool. Disperse the cells evenly by placing the

* A small bubble about twice the diameter of the original capillary is blown near the tip of the pipet. This is then drawn out to form a needle-like tip about 30 mm long. The top of the pipet is cut off about 40 mm above the tip graduation and polished. The pipet is operated by a 1 ml hypodermic syringe of the insulin type, attached to the pipet by a 50 mm piece of pressure tubing slipped part way over the syringe. A thin coating of light petroleum jelly on the plunger insures smooth operation. With a little practice, volumes of 0.02–0.2 ml can be delivered accurately and rapidly with this equipment. *Caution:* Do not allow water to get up into the barrel of the syringe, for it will prevent smooth operation of the plunger; a small cotton plug in the rubber tube will help prevent this.

thumb over the top of the tube and inverting the tube twice just before pouring it into the turbidimeter cell.

Make turbidimeter readings with a Lumetron model 402E equipped with a constant voltage transformer and a special automatic emptying cell. Use a broad band filter with a transmission peak at 5300 A and a no. 7 reduction plate over the balancing photocell because of the high absorption of the medium used. Read the tubes in either of the two following ways on the Lumetron no. 402E colorimeter. (1) Set up the instrument so that a direct reading is taken from the galvanometer scale. A constant voltage transformer is absolutely essential when direct readings are used, because the balancing photocell will not take care of all line fluctuations except when the galvanometer shows no current flowing in the balanced circuit. (2) The transmission dial is used to obtain the readings in the usual null point method. This method is much slower and does not improve the over-all precision of the test. Plot the readings obtained against the number of units in the tubes and connect the points to form a smooth curve to be used as a standard. Obtain readings on the unknown samples and compare against the standard curve, using only the portion of the curve between 0.1 and 0.5 unit. Determine the number of units in the tube. (The unitage found by this method will agree closely with the Oxford plate method unless the sample has a high percentage of penicillin K. Penicillin K is approximately 125 per cent as active in the turbidimetric test as it is on the Oxford plate test.)

2. BODY FLUIDS

Accurate determination of penicillin concentrations in the blood of animals or patients treated with various penicillin preparations has been a difficult problem. The low concentrations involved have defied chemical analyses so that bacteriologic methods remain the only recourse. Of the latter methods, two major types must be considered. (1) The broth methods, which require a large number of individual tubes in multiple series to give an adequate number of replicate analyses for accurate determination. The accuracy of these methods, whether "serial dilution" or "2 ml" dilution in type, depends also on the size of the steps in the dilution series. (2) The agar plate methods, in which measurement of the zone of inhibition of growth of an organism around a cup or paper disk filled or saturated with the test sample gives a measure of the concentration of penicillin in the sample. These methods require relatively less equipment, time and personnel than do the broth methods and allow replicate determinations on small samples. Because the concentration of a sample is determined from the measurement of inhibition zone diameters the values obtained do not depend on any fixed step-wise series of dilutions as in the broth dilution methods.

Cup-Plate Assay of Penicillin Concentrations in Plasma—S. Lutea[3]

The primary limitation in the use of the cup-plate method for blood penicillin determinations has been that 0.3 unit/ml is the minimal concentration of penicillin which would give a readable zone of inhibition with *Staph. aureus*, the organism usually employed. Starkey[4] has reported that *Sarcina lutea* exhibits a sensitivity to penicillin about 10 times that of *Staph. aureus* in cup-plate tests.

By the use of *S. lutea* as inoculating organism in place of *Staph. aureus*, the cup-plate method of assay of penicillin has been adapted to the determination of penicillin in plasma. This modification permits the determination of penicillin concentrations as low as 0.02 to 0.03 unit/ml, and, because the range of the test is wide, concentrations as high as 5–10 units/ml may be read without dilution. Five replicate values may be obtained from little more than 1.0 ml of plasma since the cups contain about 0.2 ml each.

When penicillin is added to normal plasma and assayed by the cup-plate method smaller zones are obtained than from similar concentrations of aqueous or buffer (pH 6.0) solutions of penicillin. This is probably due to (*a*) destruction of penicillin during incubation in the plasma, whose pH rises to about 8.0 or more, and (*b*) combination of penicillin with plasma proteins into a nondiffusible complex.

To eliminate these factors, the daily penicillin standards run in the test are prepared in normal plasma. Because the experimental animals were dogs, dog plasma was first used for all standard solutions and dilution of high potency experimental samples. Later it was found that identical results were obtained with normal horse plasma, which is readily available in sizable quantities and hence provides a considerable advantage in assay of the large number of samples. The assay method as now practiced follows.

PROCEDURE

Culture for inoculation.—Prepare a 48 hr culture of *S. lutea* in Bacto Yeast Beef Broth, 1.75 per cent, by continuous shaking at 27 C. This culture, properly refrigerated, has been found to be satisfactory for inoculating top agar for periods up to 4 weeks. The usual inoculation is 2 per cent of culture in a 1:1 dilution of nutrient agar. If growth is insufficient, particularly with an old culture, this may be satisfactorily increased to as high as 5 per cent.

Preparation of plates.—Use 10 ml of nutrient agar containing in each 1000 ml, 1.5 g of beef extract, 3 g of yeast extract, 6 g of peptone, 1 g of dextrose and 15 g of agar as a base medium in standard 100 ml Petri

[3] Burke, J. C., Squibb Institute for Medical Research. Unpublished data.
[4] Personal communication.

dishes. Adjust nutrient agar to pH 7.0 before autoclaving. Dilute nutrient agar with an equal volume of sterile distilled water and inoculate with 2 per cent by volume of the 48 hr culture of *S. lutea* and use 4 ml of this mixture as top agar. Set four or five 8 mm steel cups on this agar per plate and refrigerate the plates until needed. It is not advisable to hold inoculated plates more than 12 hr before use unless new standards are run at the same time, since aging of the inoculated plates tends to produce more rapid growth of the organism when incubated, and thus smaller than usual inhibition zones are obtained.

Standards.—Prepare standard solutions of crystalline sodium penicillin G (or other reference standard) in normal plasma in concentrations of 3.0, 1.0, 0.3, 0.1, 0.03 unit/ml. A daily standard curve is plotted from the average diameters of the zones obtained from five cups with each of these concentrations. If log concentration be plotted against average zone diameter, a curve only slightly concave to the base line is obtained.

Technique.—Draw 5 ml of blood aseptically into a tube containing 0.25 ml of 0.3M sodium oxalate solution. Centrifuge the blood and transfer the plasma immediately to the test plate cups. Enough plasma is easily obtained from a sample of this size to run five or more duplicate cups without dilution. If higher than 3 units/ml concentration is suspected in the plasma samples, dilute 1:5 or 1:10 with normal plasma.

Optimal temperature for growth of *S. lutea* is 28–30 C, and the incubation time of plates in this temperature is 12–16 hr. The organism, however, will grow reasonably well at somewhat lower temperatures, and satisfactory growth is generally obtained at room temperature (22–25 C) in incubation periods of 18–24 hr. Zone diameters are measured preferably with a colony counter or similar measuring device. Read penicillin concentration corresponding to the average diameter of the five test sample zones from the daily plotted standard curve.

Serial Dilution in Body Fluids—B. Subtilis[9]

PROCEDURE

Place 0.5 ml amounts of the broth (see Notes) in sterile Wassermann tubes and serially dilute by halves by adding 0.5 ml of the fluid being tested to one of the tubes and carrying 0.5 ml in serial dilution for as many tubes as necessary. The first tube in the series contains 0.5 ml of the material under test only. Prepare a standard solution containing 1 unit of penicillin per ml in broth. This 1 unit standard is diluted exactly as above in serial dilution by halves. Then 1.5 ml of a 1:100 dilution of the test organism in broth is added and all tubes are incubated at 37 C overnight. A duplicate dilution series of at least three tubes is prepared for each serum in exactly the same manner except that to each of these

[9] Randall, W. A.; Price, C. W., and Welch, H.: Estimation of penicillin in body fluids, Science 101: 365, Apr. 6, 1945; Chandler, V. L.; Price, C. W., and Randall, W. A.: Control and evaluation of blood serum assays for penicillin, Science 102: 355, Oct. 5, 1945.

tubes is added 1.5 ml of the 1:100 dilution of the test organism in broth containing sufficient sterile penicillin inactivator to destroy all of the penicillin in the serum. A control tube containing 0.5 ml of 100 units/ml standard penicillin plus 1.5 ml of the inactivator-containing inoculum should show growth.

The concentration of penicillin in the unknown is then determined by comparing the end-point of the unknown with that of the standard. In the example shown, the standard caused complete inhibition in the sixth tube. Since this represents 1 unit/ml, serum A contains twice this amount, or 2 units/ml, whereas serum B, which caused complete inhibition in the third tube, contains 0.125 unit/ml. The test as described here can be used to determine potencies as low as 0.03 unit/ml if blood-inhibitory substances do not interfere. Since the penicillin is inactivated in the duplicate control series of each specimen, lack of growth of the organism in one or more tubes reveals the titer of the blood-inhibitory substances. If the inhibition titers of the penicillin assay series and the control series are equal, as in the case of serum C, the serum under test is considered to have no penicillin activity. However, if the assay series shows one or more tubes of inhibition beyond that of the control series, the finding is recorded as the penicillin concentration of the serum.

	Tube									Control		
	1	2	3	4	5	6	7	8	9	1	2	3
Standard penicillin, 1.0 unit/ml	0	0	0	0	0	0	+	+	+			
Serum A	0	0	0	0	0	0	0	+	+	0	+	+
Serum B	0	0	0	+	+	+	+	+	+	0	+	+
Serum C	0	+	+	+	+	+	+	+	+	0	+	+

Standard 1.0 unit shows inhibition in 6 tubes.
Serum A = 2.0 units.
Serum B = 0.125 unit.
Serum C = 0.0 unit (control shows same inhibition as test).

NOTES

1. The broth employed is the same as that described for the official FDA *Staph. aureus* plate assay of penicillin (p. 4).
2. *Bacillus subtilis* SD (PCI 220) is maintained in the same broth. Eighteen hr cultures in 10 ml of broth are held in the refrigerator for daily use and may be utilized for at least 2 months.

Serial Dilution in Body Fluids—B. Subtilis Reductase Method[10]

PROCEDURE

Place 11 sterile Wassermann tubes in a rack. The first eight tubes are for the test proper; tubes 9, 10 and 11 are for penicillinase (clarase) control. To each tube, except 1 and 9, add 0.5 ml of the following medium: milk powder, 176.0 g; Trypticase (BBL), 22.0 g; agar, 1.1 g; 1 tablet of National Aniline Certified, methylene blue thiocyanate made up to 2.2 liters with distilled water (sterilize in 100 ml amounts at 10 lb for 15 min).

A series of tubes like the foregoing must be prepared for each specimen to be tested. A set of tubes is used for the standard, which is prepared by diluting a standard penicillin to contain 1.0 unit/ml. To the first tube add 0.5 ml of the diluted standard (0.5 unit). To the second tube add 0.5 ml of the standard. Mix the penicillin and medium and transfer 0.5 ml to tube 3 and so on, discarding 0.5 ml from tube 8. In another series of tubes add 0.5 ml of the specimen of blood serum or urine to each of tubes 1 and 9 and 0.5 ml to each of tubes 2 and 10. After mixing, transfer 0.5 ml from tube 2 to tube 3, etc., discarding 0.5 ml from tube 8. Then transfer 0.5 ml from tube 10 to tube 11 and discard 0.5 ml from tube 11. To each tube, 1 through 8, in the standard and test series add 1.5 ml of the culture medium which has been inoculated with 5.0 ml/100 ml of a culture of *B. subtilis*. To tubes 9 through 11 add 1.5 ml of medium plus culture plus penicillinase (0.25 ml of a 4 per cent clarase to 100 ml of medium). Tubes may be incubated at 37 C for 12–16 hr or at 20 C for 24 hr. Where growth of *B. subtilis* has occurred the milk medium in the lower two thirds or more of the tube will be reduced, i.e., white. There may be a blue color owing to atmospheric oxidation; this is of no significance. The end-point of the test is the last tube, which is still blue throughout. The end-point of the standard, indicating the concentration required to inhibit the test organism, is the last complete blue tube. (The concentration of penicillin in the unknown specimen is determined by comparison with the standard.) For example, if tube 6 of the standard is the end-point, it indicates that 0.015 unit of penicillin inhibits the test organism. If the end-point of the unknown were likewise tube 6, the unknown would have 1.0 unit/ml; if the end-point were tube 5, the concentration would be 0.5 unit/ml, and so on.

NOTES

1. Serum containing blood cells or hemolyzed specimens are unsatisfactory for use with this test because of the reducing power of hemoglobin.
2. *Bacillus subtilis* PCI 220.

[10] Reid, R. D., and Brewer, J. H.: Reductase method for determination of penicillin concentrations in body fluids, J. Bact. 52: 251, 1946.

Serial Dilution in Body Fluids—Hemolytic Streptococci[11]

PROCEDURE

Place 8–10 sterile Wassermann tubes in a rack and add 0.2 ml of veal infusion broth to all except the first tube. Prepare a similar series of tubes for each unknown to be tested. Prepare a standard control for each test by making a solution of penicillin in 0.85 per cent sodium chloride in a final concentration of 5 units/ml. Store the penicillin at 4 C when not in use. Add 0.2 ml of the standard to each of the first two tubes of one series, mix the contents of tube 2 and transfer 0.2 ml to tube 3, etc.; discard 0.2 ml from the last tube of the series. Prepare the unknown as above, adding 0.2 ml to the first two tubes of a series, and dilute serially. From a 12 hr broth culture of #98 beta hemolytic streptococcus prepare the inoculum in veal heart infusion broth containing 1 per cent human group O erythrocytes so that each milliliter contains 1000–10,000 organisms. Add 0.5 ml of the inoculum to each tube and incubate at 37 C for 18–24 hr. Determine the end-point by examining the disk of red cells at the bottom of the tube. The highest dilution which shows a disk with sharp edges and no change in color of the erythrocytes is the smallest amount of the standard or unknown which inhibits growth. This may be checked by streaking the culture in this tube on a blood agar plate. By knowing the amount of penicillin required to inhibit growth in the standard, one may determine the amount of penicillin in the unknown.

NOTES

1. In general, any strain of group A beta hemolytic streptococcus is satisfactory.
2. For accuracy, pipets should be changed in making serial dilutions, but for routine use this is not necessary. Smaller dilution increments may be used, thus increasing the sensitivity of the test.
3. For fluids or exudates known to be contaminated, sterilize by passage through a Berkefeld or Seitz filter.

Serial Dilution in Body Fluids—Hemolytic Streptococci (Phenol Red Broth)[12]

PROCEDURE

Indicator.—Phenol red gives its maximal color change between pH 7 and pH 8. At pH 6.8 it is bright yellow and at pH 7.6 cherry red. A very slight acid production, therefore, changes the color from red to yellow. The use of phenol red also permits the pH of the original medium to be between 7 and 8, which is the range suitable for growth of the test or-

[11] Rammelkamp, C. H.: Method for determining concentration of penicillin in body fluids and exudates, Proc. Soc. Exper. Biol. & Med. 51: 95, October, 1942.

[12] Fleming, A., and Smith, C.: Estimation of penicillin in serum, Lancet 1: 401, Mar. 29, 1947.

Serial Dilution in Body Fluids—B. Subtilis Reductase Method[10]

PROCEDURE

Place 11 sterile Wassermann tubes in a rack. The first eight tubes are for the test proper; tubes 9, 10 and 11 are for penicillinase (clarase) control. To each tube, except 1 and 9, add 0.5 ml of the following medium: milk powder, 176.0 g; Trypticase (BBL), 22.0 g; agar, 1.1 g; 1 tablet of National Aniline Certified, methylene blue thiocyanate made up to 2.2 liters with distilled water (sterilize in 100 ml amounts at 10 lb for 15 min).

A series of tubes like the foregoing must be prepared for each specimen to be tested. A set of tubes is used for the standard, which is prepared by diluting a standard penicillin to contain 1.0 unit/ml. To the first tube add 0.5 ml of the diluted standard (0.5 unit). To the second tube add 0.5 ml of the standard. Mix the penicillin and medium and transfer 0.5 ml to tube 3 and so on, discarding 0.5 ml from tube 8. In another series of tubes add 0.5 ml of the specimen of blood serum or urine to each of tubes 1 and 9 and 0.5 ml to each of tubes 2 and 10. After mixing, transfer 0.5 ml from tube 2 to tube 3, etc., discarding 0.5 ml from tube 8. Then transfer 0.5 ml from tube 10 to tube 11 and discard 0.5 ml from tube 11. To each tube, 1 through 8, in the standard and test series add 1.5 ml of the culture medium which has been inoculated with 5.0 ml/100 ml of a culture of *B. subtilis*. To tubes 9 through 11 add 1.5 ml of medium plus culture plus penicillinase (0.25 ml of a 4 per cent clarase to 100 ml of medium). Tubes may be incubated at 37 C for 12–16 hr or at 20 C for 24 hr. Where growth of *B. subtilis* has occurred the milk medium in the lower two thirds or more of the tube will be reduced, i.e., white. There may be a blue color owing to atmospheric oxidation; this is of no significance. The end-point of the test is the last tube, which is still blue throughout. The end-point of the standard, indicating the concentration required to inhibit the test organism, is the last complete blue tube. (The concentration of penicillin in the unknown specimen is determined by comparison with the standard.) For example, if tube 6 of the standard is the end-point, it indicates that 0.015 unit of penicillin inhibits the test organism. If the end-point of the unknown were likewise tube 6, the unknown would have 1.0 unit/ml; if the end-point were tube 5, the concentration would be 0.5 unit/ml, and so on.

NOTES

1. Serum containing blood cells or hemolyzed specimens are unsatisfactory for use with this test because of the reducing power of hemoglobin.
2. *Bacillus subtilis* PCI 220.

[10] Reid, R. D., and Brewer, J. H.: Reductase method for determination of penicillin concentrations in body fluids, J. Bact. 52: 251, 1946.

Serial Dilution in Body Fluids—Hemolytic Streptococci[11]

PROCEDURE

Place 8–10 sterile Wassermann tubes in a rack and add 0.2 ml of veal infusion broth to all except the first tube. Prepare a similar series of tubes for each unknown to be tested. Prepare a standard control for each test by making a solution of penicillin in 0.85 per cent sodium chloride in a final concentration of 5 units/ml. Store the penicillin at 4 C when not in use. Add 0.2 ml of the standard to each of the first two tubes of one series, mix the contents of tube 2 and transfer 0.2 ml to tube 3, etc.; discard 0.2 ml from the last tube of the series. Prepare the unknown as above, adding 0.2 ml to the first two tubes of a series, and dilute serially. From a 12 hr broth culture of #98 beta hemolytic streptococcus prepare the inoculum in veal heart infusion broth containing 1 per cent human group O erythrocytes so that each milliliter contains 1000–10,000 organisms. Add 0.5 ml of the inoculum to each tube and incubate at 37 C for 18–24 hr. Determine the end-point by examining the disk of red cells at the bottom of the tube. The highest dilution which shows a disk with sharp edges and no change in color of the erythrocytes is the smallest amount of the standard or unknown which inhibits growth. This may be checked by streaking the culture in this tube on a blood agar plate. By knowing the amount of penicillin required to inhibit growth in the standard, one may determine the amount of penicillin in the unknown.

NOTES

1. In general, any strain of group A beta hemolytic streptococcus is satisfactory.
2. For accuracy, pipets should be changed in making serial dilutions, but for routine use this is not necessary. Smaller dilution increments may be used, thus increasing the sensitivity of the test.
3. For fluids or exudates known to be contaminated, sterilize by passage through a Berkefeld or Seitz filter.

Serial Dilution in Body Fluids—Hemolytic Streptococci (Phenol Red Broth)[12]

PROCEDURE

Indicator.—Phenol red gives its maximal color change between pH 7 and pH 8. At pH 6.8 it is bright yellow and at pH 7.6 cherry red. A very slight acid production, therefore, changes the color from red to yellow. The use of phenol red also permits the pH of the original medium to be between 7 and 8, which is the range suitable for growth of the test or-

[11] Rammelkamp, C. H.: Method for determining concentration of penicillin in body fluids and exudates, Proc. Soc. Exper. Biol. & Med. 51: 95, October, 1942.

[12] Fleming, A., and Smith, C.: Estimation of penicillin in serum, Lancet 1: 401, Mar. 29, 1947.

ganisms. Add sufficient phenol red to give a distinct red color to the medium when observed in a fine capillary tube.

Test organism.—A hemolytic streptococcus is used. Use an inoculum of 5 mm³ of a 24 hr broth culture to 1 ml of the medium. There is considerable latitude in this direction.

Medium.—Human serum is preferable, but horse, sheep or ox serum can be used. The medium can be made up in bulk, steamed and distributed in small volumes, or small quantities of 5–10 ml can be made at

Serum	2.00 ml
10% glucose solution	2.00 ml
Distilled water	6.00 ml
Phenol red, saturated solution	0.25 ml

a moment's notice and boiled for 1 or 2 min over a Bunsen flame. This is an advantage in a hospital laboratory, where small quantities of human serum are always available. For making small quantities it is convenient to keep 10 per cent glucose solution in sealed 1 ml ampules which have been autoclaved.

Technique.—Inoculate a suitable amount of the medium with the test organism. Make serial dilutions of the serum to be tested (25 mm³ volumes) in the inoculated medium on the surface of a paraffined slide. Each of these drops is touched with the end of a capillary tube, and the fluid runs into the tube, which is sealed (sealing is not essential). Run the fluid toward the center of the capillary tube by tilting. Place horizontally on a plasticine-covered slide, where it remains in position, and incubate. By this method the strongest concentration of the test serum is 1:2. If it is desired to use almost undiluted serum, 25 mm³ of undiluted serum can be mixed with 5 mm³ of 50 per cent serum water containing 5 per cent glucose and sufficient phenol red and inoculated with five times as many streptococci.

After incubation the tubes in which the streptococci have grown are bright yellow and show a heavy precipitate. There is often an intermediate tube in which there is the color change, but sufficient acid has not been produced to precipitate the serum. The tubes in which growth has been prevented by penicillin are red or red-violet. The end-point is sharp, and the results are easily read.

Serial Dilution in Body Fluids—Hemolytic Streptococci (Capillary Tubes)[13]

PROCEDURE

Unknown.—Set up eight small test tubes (Wassermann) in a suitable rack. To each, except the first, add 0.2 ml of physiologic saline solution. To tubes 1 and 2 add 0.2 ml of the unknown whole (citrated) blood.

[13] Wolohan, M. B., and Cutting, W. C.: Simple technique for estimation of penicillin in blood and other body fluids, J. Lab. & Clin. Med. 30: 161, February, 1945.

From tube 2 transfer 0.2 ml to tube 3, then from tube 3 transfer 0.2 ml to tube 4, and so on, to make serial dilutions. Discard 0.2 ml from the final tube.

To each of the eight tubes add 0.2 ml of a suspension of hemolytic streptococci in broth prepared as follows: Transfer streptococci from a 24 hr culture in broth, by loop, to fresh broth. Use one loop of streptococci for each milliliter of broth and prepare enough for all the unknown and control blood samples to be assayed.

Shake each tube, hold almost horizontally, and from it, by capillarity, nearly fill a glass capillary tube about 6 cm long. While still holding the capillary tube horizontally, embed the end in a layer of modeling clay filling a groove in a small board. When all the capillary tubes are in place in a row, turn the board so that the tubes are upright. Incubate for 24 hr at 37 C, then inspect the tubes for presence of hemolysis and colonies of streptococci.

Control.—Set up a series of tubes, as for the unknown blood, but use defibrinated horse blood containing 1 unit of penicillin per ml instead of the unknown blood sample.

Estimation.—The most dilute control capillary tube without hemolysis *and bacterial colonies indicates the dilution of 1 unit of penicillin which* will inhibit the inoculum of streptococci. The preceding tube indicates the dilution of 0.5 unit which will inhibit the streptococci, and so forth. By comparison of the end-point in the control series with that in the unknown series, the concentration of penicillin in the latter is estimated. Thus, if the end-point in the control series is in tube 6, as it usually is, the unknown contains 1 unit if it also gives the end-point in tube 6; 0.5 if in tube 5, and so forth.

NOTES

1. Sterilized glassware is used, but the tubes and pipets are not flamed, except initially. To simplify preparation, the test tubes (Wassermann) are placed, without plugging, in a large wire rack which is then wrapped in paper and sterilized. A small tear in one corner allows removal of individual tubes as needed. Any capillary tubes may be used, but the Kimble no. 34500, for melting point determinations, has been most satisfactory.

Although the concentration of red cells differs in each tube, this does not affect the accuracy of the test. In this procedure the centrifuging of blood specimens and addition of known amounts of red cells are eliminated. Simplification of the procedure, therefore, depends on these omissions and on use of capillary tubes. Rabbit blood occasionally gives results in which the end-point is difficult to determine, presumably because of delay in red cell sedimentation. Because penicillin is stated to enter red blood corpuscles only in minute amounts, a considerable shift in hematocrit would affect the concentration of penicillin when determined in whole blood, as in this procedure. However, the error seldom is sig-

nificant, especially when considered in the light of the large inherent error of the serial dilutions.

2. The concentration of penicillin in other body fluids may be determined similarly. Urine must be diluted before estimation to bring the usually high urinary penicillin levels into the range of blood levels. It is usually diluted with 9 parts of defibrinated horse blood, but broth may be used (the end-point is then determined only on the basis of colony growth). The final values must, of course, be corrected for the dilution factor.

3. Age of the streptococci (up to 5 days), their dilution between 10^{-6} and 10^{-4} and the exact period over which the test is allowed to run have practically no effect on the results.

ASSAY OF PENICILLIN POTENCY: B. CHEMICAL AND PHYSICAL METHODS

Colorimetric Method—Penicillin Powder[14]

PROCEDURE

To 5 ml of a glycine buffer pH 2.0 add 25 ml of reagent grade chloroform and 5 ml of an aqueous solution containing 20–120 μg of penicillin. Immediately upon addition of penicillin to the cold solutions shake the system vigorously for 2 min. Allow the phases to separate and run the chloroform into a chilled 25 ml glass-stoppered graduate containing 3 g of anhydrous sodium sulfate. To 15 ml of benzene containing 10 mg of the dye, N-(1-naphthyl-4-azobenzene)-ethylenediamine, add 20 ml of the superficially dried chloroform extract and immediately thereafter 5 ml of a solution of 5 ml of glacial acetic acid in 1 liter of benzene. Allow the reaction mixture to stand at room temperature in a closed container for 3 hr (±3 min). Shake vigorously for 10 sec with 25 ml of 0.05N sodium hydroxide in a 125 ml separatory funnel. When both phases have completely separated and are crystal clear, draw off the lower layer, leaving 0.2–0.5 ml of the organic solvent in the funnel. (During this phase separation, avoid disturbance of the interfacial material.) Repeat the extraction with 25 ml of chloroform and after complete phase separation draw off the chloroform layer as before, leaving 0.2–0.3 ml in the funnel. After addition of 1 ml of concentrated hydrochloric acid, followed by 15 ml of a mixture of 1 volume of butanol and 4 volumes of benzene, shake the system for 10 sec to transfer the red condensation product to the butanol-benzene phase. To 10 ml of the butanol-benzene extract add 2 ml of a mixture of 5 volumes of concentrated hydrochloric acid and 95 volumes of absolute ethanol. (The strong acid is required to bring about maximal tautomerism.) Measure the color intensity in a photoelectric colorimeter equipped with a no. 540 filter and determine the concentration of penicillin from a calibration curve obtained by subjecting a number of standard solutions of different concentrations of penicillin to the foregoing procedure.

[14] Scudi, J. V.: Colorimetric method for determination of penicillin, J. Biol. Chem. 164: 183, 1946.

Colorimetric Method—Penicillin Broth[14]

PROCEDURE

Treat samples of broth, selected at random, with filter aid (0.5 g of Hyflo Super-Cel to 50 ml of broth) and shake the mixture well. Filter the broth through a Buchner funnel precoated with a thin layer of Super-Cel. Aliquots of the filtrate (1–6 ml, depending on potency of the broth) are then analyzed by the foregoing procedure outlined for powder after adjusting to volume. This gives the "total" value. Treat two equal aliquots with 0.4N sodium hydroxide to give solutions 0.2N in alkali. Allow these aliquots to stand at room temperature for 1–2 hr to inactivate the penicillin. Adjust to volume and complete the analyses as usual.

NOTES

1. The buffer is prepared from 600 ml of 0.2M glycine in 0.2M sodium chloride by addition of 0.2M hydrochloric acid to pH 2.0.
2. Crystalline samples of penicillin G sodium salt may be weighed with the usual precautions, but commercial samples should be finely divided and should be rapidly weighed in a dry atmosphere.
3. All solutions must be kept cold (0–5 C) throughout the procedure.
4. Preparation of the dye is described in the original publication.

Alkalimetric Method[15]

PROCEDURE

Transfer 10 ml of an aqueous solution containing 60,000–150,000 units of penicillin to a 50 ml beaker. Immerse the tips of a glass and calomel electrode in the solution and introduce a mechanical stirrer. Connect the electrodes to a suitable pH meter and start the stirrer. *Bring the solution to pH 8.0 by adding dropwise 0.1N NaOH, then add* exactly 10.0 ml of 0.1N NaOH, which will bring the pH to approximately 12. Allow the solution to stir at room temperature for 10–12 min, then titrate back to pH 8.0 with 0.1N HCl.

Calculation:

Units sodium penicillin = (ml of 0.1N NaOH − ml of 0.1N HCl) × 59,400

NOTES

1. The factor 59,400 is based on a molecular weight of 356 for pure sodium penicillin G and an established potency of 1667 units/mg.
2. This method is applicable to relatively low potency material except when high buffering action or other caustic sensitive compounds are present.
3. It is advantageous to standardize against a sample of penicillin of

[15] Charles Pfizer & Company, Inc., Brooklyn. Unpublished data.

known potency as the reaction is influenced by too great variations in room temperature.

Alkalimetric Method Using Hydrogen Peroxide[16]

PROCEDURE

Transfer an aliquot containing 30,000–150,000 units of penicillin to a 50 ml beaker equipped with a glass and calomel electrode of a suitable pH meter and dilute with distilled water to 20 ml. A motor-driven stirrer may be used but is not essential. Adjust with N/50 sodium hydroxide to pH 8.0. Add 3 ml of 3 per cent hydrogen peroxide previously adjusted to pH 8.0. The pH of the solution drops quickly. Immediately add 0.02N sodium hydroxide dropwise, maintaining the pH of the solution between 7.9 and 8.3. When the pH drop is less than 0.2 unit in 40 sec, record the volume of alkali used after addition of the hydrogen peroxide. This determination requires approximately 20 min.

Calculation:

$$\text{ml of } 0.02N \text{ NaOH} \times 7.12 = \text{mg of sodium penicillin in aliquot}$$

NOTE

This method is similar in applicability to the foregoing alkalimetric assay with the added advantage of utilizing a somewhat smaller sample and a more dilute sodium hydroxide solution for titration.

Titration by Iodometric Method[17]

PROCEDURE

Dilute a weighed sample (approximately 30 mg) with 1 per cent phosphate buffer pH 6.0 to a concentration of approximately 1.2 mg/ml (2000 units/ml). Add 2.0 ml aliquots to each of two 125 ml glass-stoppered Erlenmeyer or iodine flasks. To one add 2.0 ml of 1N NaOH and allow to stand at room temperature for 15 min. At the end of this time add 2.0 ml of 1.2N HCl and add 10.0 ml of 0.01N I_2 (prepared from 0.1N I_2 U.S.P.). (Equal volumes of 1N NaOH and 1.2N HCl, when mixed, give pH 1.0.) After 15 min titrate the excess iodine, using 0.01N $Na_2S_2O_3$ (prepared from 0.1N $Na_2S_2O_3$) standardized accurately against potassium iodate. Toward the end of the titration add approximately 5 ml of CCl_4. Continue the titration by the addition of 0.01–0.02 ml portions of the 0.01N $Na_2S_2O_3$, shaking vigorously after each addition. The end-point is reached when the CCl_4 layer becomes colorless. To the second flask add 10 ml of the 0.01N I_2 and titrate immediately with

[16] Charles Pfizer & Company, Inc., Brooklyn. Unpublished data.
[17] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

0.01N $Na_2S_2O_3$ for the blank determination. Regard the difference in titers divided by 2.52 as the milligrams of penicillin sodium salt.

Titration Using Penicillinase[18]

PROCEDURE

Prepare an aqueous solution of penicillinase so that each milliliter of solution will contain sufficient enzyme to inactivate completely 20,000 units of penicillin in 10 min or less. Adjust this solution to pH 8.0 shortly before use, using one set of electrodes (glass and calomel) with a suitable pH meter. Similarly, adjust the penicillin solution containing 10,000–20,000 units in approximately 10 ml water to pH 8.0, using a second set of electrodes. (This set should be used only for the adjusting of penicillin solutions and should never come in contact with the penicillinase solution.) Raise the electrodes just above the surface of the adjusted penicillin solution and rinse them with small volumes of water. Place the penicillin solution under the first set of electrodes and add 1 ml of the pH 8.0 penicillinase solution. (The pH falls rapidly.) Readjust to maintain a pH of approximately 6.8 by gradual addition from a microburet of 0.02N NaOH. When the pH becomes constant, complete titration by adding 0.02N NaOH until pH 8.0 is obtained. The total volume of 0.02N NaOH used times 11,870 gives the units of penicillin in the sample. The figure 11,870 is a constant for sodium penicillin G.

NOTE

Penicillinase is an enzyme obtained from certain bacteria capable of inactivating penicillin. The enzyme should be of such potency that not more than 100 mg of the dry enzyme is required to inactivate completely in 10 min or less, 20,000 units of penicillin contained in 10 ml of water.

Fluorometric Method[19]

PROCEDURE

To 8 ml of an aqueous solution containing 0.0625–0.625 μg of penicillin per ml, add successively 12 ml of reagent chloroform and 2 ml of a Sörensen glycine buffer at pH 2.0. Shake the mixture vigorously for 30 sec and allow the phases to separate completely. Draw off the chloroform layer into a chilled, glass-stoppered graduate and dry rapidly with 1–2 g of anhydrous sodium sulfate. To 5 ml of benzene containing 10 mg of 2-methoxy-6-chloro-9-(B-amino-ethyl)-aminoacridine add successively 2 ml of reagent grade acetone, 10 ml of the chloroform-penicillin extract and 5 ml of a solution containing 10 ml of glacial acetic acid in 1 liter of

[18] Murtaugh, J. J., and Levy, G. B.: Penicillinase method for determination of potency of penicillin, J. Am. Chem. Soc. 67: 1042, 1945.

[19] Scudi, J. V., and Jelinek, V. C.: Rapid micromethod for fluorometric determination of penicillin, J. Biol. Chem. 164: 195, July, 1946.

benzene. Allow the reaction mixture to stand in a closed container at room temperature in the absence of light for 1 hr (± 5 min).

At the end of this time the extractions are begun; they should be completed as rapidly as possible with minimal exposure to light. A shaded hood is suitable for this purpose. Shake the reaction mixture vigorously for 10 sec in a 125 ml separatory funnel with 10 ml of 0.5N sodium hydroxide. After the phases have completely separated, remove the lower, organic layer. Shake the alkaline solution vigorously for 5 sec with two successive 5 ml portions of chloroform, drawing off the chloroform layer after each phase separation. Acidify the alkaline solution with 1 ml of glacial acetic acid and extract the condensation product from the reaction mixture with 15 ml of butanol-benzene solution (1:2 by volume) by shaking vigorously for 30 sec. Discard the aqueous layer and shake the organic layer for 30 sec with 10 ml of 5 per cent aqueous acetic acid. Discard the aqueous layer and add 50 ml of chloroform followed by 15 ml of 0.5N sodium hydroxide. Transfer the condensation product to the aqueous phase by shaking for 30 sec. Discard the lower organic layer and add 1 ml of concentrated hydrochloric acid to the alkaline solution.

Place the acidic solution in the cuvet and measure the fluorescence intensity in a fluorophotometer with a no. 5113 Corning glass filter, 2 mm thick, placed in the path of the incident beam and a no. 3385 Corning glass filter, 2 mm thick, placed between the sample and the photocell. Before a series of measurements is made, the fluorophotometer must be adjusted to a constant light intensity. The fluorescence of a standard solution (0.8 μg of the aminoacridine in 10 ml of 4N hydrochloric acid) is arbitrarily adjusted to 60 per cent of the galvanometer scale. Check the instrument setting before and after reading unknown solutions.

It is necessary to subtract from the final readings the values obtained in blank determinations. It is preferable to perform blank determinations with each series of analyses. By subjecting a series of standard solutions containing 0.0025–0.025 μg of penicillin per ml to the foregoing procedure, determine the average galvanometer deflection per microgram of penicillin. From the galvanometer deflection obtained for the unknown solution, calculate the amount of penicillin in the unknown.

NOTES

1. All solutions must be kept cold (0–5 C) throughout the procedure.
2. The glycine buffer is prepared from 600 ml of 0.2M glycine in 0.2M sodium chloride by the addition of 0.2M hydrochloric acid to pH 2.0.
3. Preparation of the dye is described in the original publication.

Polariscopic Method[10]

PROCEDURE

Dissolve 180 mg of sodium penicillin in 10.0 ml of freshly boiled and cooled distilled water at 25 C. Determine the specific rotation within 10 min and calculate specific rotation and units per milligram as follows:

$$\text{specific rotation} = \frac{\text{angular rotation} \times \text{volume of solution}}{\text{length tube in dcm} \times \text{g sample in solution}}$$

$$\frac{\text{specific rotation of sample}}{\text{specific rotation of pure sodium penicillin G}} \times 1667 = \text{u/mg}$$

NOTES

1. Pure sodium penicillin G has a specific rotation of 298° (±3°) at 25 C, at a concentration of 180 mg in 10 ml.

2. Applicability of the polarimetric determination is chiefly limited by the possible presence of compounds possessing optical activity, as often found in yellow amorphous penicillin samples. It gives excellent correlation with the biochemical values when applied to crystalline penicillin samples.

Penicillin G—N-ethyl Piperidine[11]

REAGENTS

The reagents described here are freshly prepared every 3 days and are of such quality that when used in this procedure with a known penicillin G not less than 97 per cent of penicillin G is recovered.

Amyl acetate solution.—Saturate amyl acetate with the N-ethyl piperidine salt of penicillin G by adding 2 mg of the salt for each 1 ml of the solvent. Cool this solution to 0–8 C and filter by drawing it through a plug of cotton on the tip of a pipet immediately before use.

Acetone solution.—Saturate reagent grade acetone with the N-ethyl piperidine salt of penicillin G, using 3 mg of salt for each 1 ml of acetone. Cool this solution to 0–8 C and filter by drawing it through a plug of cotton on the tip of a pipet immediately before use.

N-ethyl piperidine solution.—N-ethyl piperidine should be stored in brown bottles in a refrigerator. Dilute 1.0 ml of this reagent with 4.0 ml of amyl acetate. Saturate the solution with the N-ethyl piperidine salt of penicillin G, using about 3 mg of the salt for each 1.0 ml of solution. Cool this solution to 0–8 C and filter by drawing it through a plug of cotton on the tip of a pipet immediately before use.

Phosphoric acid solution.—Prepare by dissolving 1.0 ml of reagent grade phosphoric acid (85 per cent) in 4.0 ml of water. Cool to 0–8 C and shake before using.

[10] Charles Pfizer & Company, Inc., Brooklyn. Unpublished data.
[11] Sheehan, J. C.; Mader, W. J., and Cram, D. J.: Chemical assay method for penicillin G, J. Am. Chem. Soc. 68: 2407, 1946.

Sodium sulfate.—Use powdered anhydrous reagent grade sodium sulfate.

PROCEDURE

Add 4 ml of distilled water for each 200,000 units or 120 mg of the sample to be tested. Pipet a 2.0 ml aliquot into a glass test tube of about 10 ml capacity and cool to 0–5 C. Add 2 ml of the amyl acetate solution and 0.5 ml of the phosphoric acid solution, stopper and shake vigorously for approximately 15 sec. Centrifuge to obtain a clear separation of the two layers (approximately 20 sec). After centrifuging, remove as much of the amyl acetate layer as possible (usually about 1.7–1.8 ml) with a 2 ml hypodermic syringe equipped with a suitable needle. Place about 0.1 g of the sodium sulfate in a microfilter funnel (approximately 10 mm diameter) having a fritted glass disk of medium porosity and add the amyl acetate solution from the hypodermic syringe. Collect the filtrate by suction in a small test tube which has been placed in a suction flask. Surround the suction flask with cracked ice. Pipet a 1.0 ml aliquot of the amyl acetate filtrate into a tared flat bottom glass tube (approximately 15 × 50 mm) containing 1.0 ml of the acetone solution and 0.5 ml of the N-ethyl piperidine solution. The time elapsing between acidification and addition of the filtrate to the reagents should not be more than 3 min. Place the glass tube containing this mixture in a large weighing bottle, stopper and allow to stand for not less than 2 hr in a refrigerator at 0–8 C. Remove the liquid from the precipitate by means of a tared microfilter stick and wash with a total of 1 ml of the acetone solution, adding it by means of a hypodermic syringe equipped with a fine needle. Place the filter stick inside the glass tube, dry under vacuum at room temperature for not less than 1 hr and weigh. (Save all N-ethyl piperidine penicillin G residues for saturating reagents.) Remove a 1.0 ml aliquot of the original aqueous penicillin solution and dilute to 25.0 ml (approximately 2000 units/ml) with 1 per cent phosphate buffer pH 6.0. Using 2.0 ml aliquots of this dilution, determine the amount of penicillin in the original solution in mg/ml by the iodometric assay procedure (p. 35).

$$\%\text{ of penicillin G} = \frac{\text{wt. in mg N-ethyl piperidine penicillin ppt} \times 159.3}{\text{mg of penicillin in 2.0 ml of original sol.}}$$

Penicillin G—Spectrophotometric Method[22]

The wide range of potency encountered in commercial penicillin products necessitates the use of different procedures, depending on the nature of the sample being assayed. In high purity crystalline penicillin products (95 per cent or greater), the G content is determinable directly on a dilution of the solution assayed polarimetrically.

[22] Charles Pfizer & Company, Inc., Brooklyn. Unpublished data.

PROCEDURE A

On the basis of the polarimetric assay, dilute the solution to a concentration equivalent to 1.8 mg of pure sodium penicillin per ml. Transfer an aliquot to a 1 cm quartz cell, determine the optical density at 263 μ and subtract from it the value obtained at 280 μ. Where the latter value is less than 0.10, the difference obtained is directly indicative of the G content. Pure sodium penicillin G gives a 263–280 μ difference of 0.79, whereas the "non-G" nonaromatic penicillins (species F and K) give a value of 0.12. The assay of mixtures of pure G, pure F species and pure K species shows the optical density difference to be arithmetical, and the G content of the mixture is determined from a chart where percentage "G" is plotted against the 263 optical density minus 280 optical density value.

NOTE

Penicillin X interferes, even when present in small amounts, because it has a very high absorption at 280 μ.

PROCEDURE B

When the potency is found to fall in the 1300–1600 unit/mg range for either amorphous or crystalline penicillin, recourse is had to a preliminary purification procedure.

Add 1.8 g of ammonium sulfate A.R. and 1 drop of 3 per cent ammonium hydroxide to each 5 ml of solution used in the polariscopic assay. Cool slowly to −5 C while stirring and hold at this temperature 1 hr. Filter the crystalline slurry through a precooled semimicro Buchner funnel and wash the filter cake with 1–3 ml of ice-cold 40 per cent ammonium sulfate solution.

Dissolve the precipitate in 5 ml of water and determine the penicillin concentration polarimetrically. Dilute to a concentration equal to 1.8 mg of sodium penicillin G per ml and assay for G spectrophotometrically, as described in procedure A.

NOTES

1. Considerably more latitude is permissible in the 280 μ optical density values in the ammonium salt solution because interfering compounds are eliminated in the purification step.

2. In relatively high potency penicillin G samples the recrystallization procedure results in isolation of a product containing practically the same proportions of the various species as are present in the original sample.

PROCEDURE C

In applying the ammonium salt purification procedure to 800–1300 unit/mg potency samples, a larger sample is best employed and considerable care taken to insure a high recovery of product.

Utilizing the same general procedure as outlined in B, isolate the ammonium salt by the addition of sufficient ammonium sulfate to give a 34 per cent solution.

NOTE

Isolation of a sufficiently pure ammonium salt for spectrophotometric assay from material in the 800–1300 unit/mg potency range requires considerable variation in technique to adapt the methods to the sample.

Penicillin X[23]

PROCEDURE

Dissolve the contents of a 100,000 unit ampule of penicillin in about 20 ml of ice-cold distilled water. Transfer quantitatively to a 100 ml volumetric flask, rinsing the ampule with small portions of ice-cold water and make to 100 ml. Pipet a 50.0 ml aliquot into a 125 ml separatory funnel, then add 50.0 ml of cold chloroform and shake the mixture. Add an amount of approximately 1N H_2SO_4 to bring the pH of the aqueous layer to 2.0. (The amount of 1N H_2SO_4 to be added is calculated by titrating a separate 5.0 ml aliquot of 100 ml dilution to pH 2.0 using a suitable pH meter.) Shake the mixture vigorously for 1 min. Allow the layers to separate and filter the chloroform through a small pledget of cotton, moistened with chloroform, into a second 125 ml separatory funnel. Shake the acid aqueous solution with a second 50.0 ml of cold chloroform and, when the layers have separated, withdraw the chloroform through the same filter into the second separatory funnel. Immediately neutralize the acid aqueous solution, containing the penicillin X, with 0.1N NaOH to pH 6.5–7.0, using a pH meter, and make to 100 ml with water. Make appropriate dilutions in 1 per cent phosphate buffer pH 6.0 and assay by the cup-plate method (p. 7). Shake the combined chloroform extracts containing any penicillin G, etc., with small successive portions of cold $NaHCO_3$ solution (0.1 per cent) until the combined $NaHCO_3$ extracts give a pH of 7.0 and make to 100 ml with water. Make the proper estimated dilutions in 1 per cent phosphate buffer at pH 6.0. Assay these last dilutions by the cup-plate method. The potency of the penicillin X fraction plus potency of the penicillin G, etc., fraction should approximate that of the original solution. All of the aforementioned extractions should be carried out in a cold room.

Penicillin K[24]

PROCEDURE

Dilute a weighed sample of the contents of a vial with 0.3M phosphate (Na_2HPO_4 and KH_2PO_4) buffer pH 6.0 to give a solution containing approximately 1000 units/ml. In the case of calcium penicillin, where a precipitate of calcium phosphate occurs, remove the precipitate by filtra-

[23] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

[24] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

tion and use the clear filtrate. Place a 15.0 ml aliquot of this solution in a 125 ml separatory funnel, add 30.0 ml of chloroform U.S.P. and shake for 1 min. (Carry out all operations at room temperature.) Allow the mixture to stand with occasional swirling to settle the droplets of chloroform until the top layer is clear (usually about 10 min). Draw off all but about 2 ml of the lower chloroform layer through a small pledget of cotton into a glass stoppered flask. Take a 4.0 ml aliquot of the original solution, a 4.0 ml aliquot of the buffer solution remaining in the separatory funnel and a 10.0 ml aliquot of the chloroform solution and determine the mg/ml of penicillin in each by the iodometric assay procedure (p. 35) using 4.0 ml of the 1N NaOH and 4.0 ml of the 1.2N HCl for each of the above aliquots. Make blank determinations on the same size aliquots. Calculate the percentage of penicillin in the buffer layer and in the chloroform layer as compared to the original solution. The sum of these percentages should be 100 per cent ($\pm$ 2 per cent).

$$\%\text{ of penicillin K} = (06.92 + \%\text{ in chloroform} - \%\text{ in buffer}) \times 1.67$$

(The factors in the formula are based on distribution coefficients of penicillin K and G between chloroform and aqueous phosphate buffer at pH 6.0.)

ASSAY OF STREPTOMYCIN POTENCY: A. BIOLOGIC METHODS

1. COMMERCIAL PREPARATIONS

Cylinder-Plate Assay[25]

PROCEDURE

a) Cylinders.—Use those described under penicillin plate assay (p. 4).

b) Culture medium.—Using ingredients that conform to the standards prescribed by the U.S.P. or N.F., make nutrient agar for the seed and base layers:

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Distilled water, q s.	1000.0 ml

pH 7.8–8.0 after sterilization.

c) Working standard.—Keep the working standard (obtained from the Food and Drug Administration) constantly in the refrigerator at 15 C (59 F) or below in tightly stoppered vials, which in turn are kept in larger stoppered tubes containing anhydrous calcium sulfate. Weigh out carefully, in an atmosphere of 50 per cent relative humidity or less, appropriate amounts of the working standard and dilute in 0.05M potassium phosphate buffer pH 6.0. Keep this stock solution at 15 C; do not use it later than 30 days after it is made.

d) Standard curve.—Prepare daily in 0.10M potassium phosphate buffer (pH 7.8–8.0) a 20 μg/ml solution from the stock solution described above. Transfer to ten 100 ml volumetric flasks, containing the same buffer, the required quantities of this 20 μg/ml solution to give 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4 and 1.5 μg/ml solutions. A total of 27 plates is used in preparation of the standard curve, three for each solution except the 1.0 μg/ml solution. The last concentration is used as the reference point and is included on each plate. On each of three plates fill three cylinders with the 1.0 μg/ml standard and the other three cylinders with the concentrations under test. Thus there will be eighty-one 1 μg determinations and nine determinations for each of the other points on the curve. After the plates have incubated read the diameters of the

[25] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

circles of inhibition. Average the readings of the 1.0 μg/ml concentration and those of the point tested for each set of three plates and average also all 81 readings of the 1.0 μg/ml concentration. The average of the 81 readings of the 1.0 μg/ml concentration is the correction point for the curve. Correct the average value obtained for each point to the figure it would be if the 1.0 μg/ml reading for that set of three plates were the same as the correction point. Thus if, in correcting the 0.8 unit concentration, the average of the 81 readings of the 1.0 μg/ml concentration is 16.5 mm and the average of the 1.0 μg/ml concentration of this set of three plates is 16.3 mm, the correction is 0.2 mm. If the average reading of the 0.8 μg/ml concentration of these same three plates is 15.9 mm, the corrected value is then 16.1 mm. Plot these corrected values, including the average of the 1.0 μg/ml concentration, on two cycle semilog paper, using the concentration in μg/ml as the ordinate (logarithmic scale) and the diameter of the zone of inhibition as the abscissa. Draw the standard curve through these points. The 10 points selected to determine the curve are arbitrary and should be so chosen that the limits of the curve will fill the needs of the laboratory. However, the potency of the sample under test should fall in the interval of from 60 to 150 per cent of the correction point of the standard curve.

e) Preparation of sample.—Dissolve volumetrically in sterile distilled water the sample to be tested to make a convenient stock solution. Further dilute this solution volumetrically to contain 100 μg of streptomycin base (estimated) per ml. Transfer 1.0 ml of this 100 μg/ml (estimated) solution to a 100 ml flask and make up to volume with 0.10M potassium phosphate buffer pH 7.8–8.0. Use this last dilution in the assay for potency.

f) Preparation of spore suspension.—The test organism is *Bacillus subtilis* (ATCC 6633). Maintain the test organism on nutrient agar prepared as described in (*b*). Prepare a spore suspension by growing the organism in Roux bottles on agar of this same composition for one week at 37 C. Suspend the spores in sterile distilled water and heat for 30 min at 65 C. Wash the spore suspension three times with sterile distilled water, heat again for 30 min at 65 C and resuspend in sterile distilled water. Maintain the spore suspension at approximately 15 C. Determine by appropriate tests the quantity of spore suspension to be added to each 100 ml of agar for the secondary layer that will give sharp clear zones of inhibition.

g) Preparation of plates.—Add 21 ml of agar (paragraph (*b*)) to each Petri dish (20 × 100 mm). Melt the agar to be used for the secondary layer, cool to 55–60 C and add the spore suspension (paragraph (*f*)). Mix thoroughly and add 4 ml to each of the plates containing the 21 ml of uninoculated agar. Tilt the plates back and forth to spread the inoculated agar evenly over the surface. Refrigerate until ready to add streptomycin (at least 1 hr).

h) Plate assay.—Place six cylinders on the inoculated agar surface so

that they are at approximately 60° intervals on 2.8 cm radius. Use three plates for each sample. Fill three cylinders on each plate with the 1.0 μg/ml standard and three cylinders with the 1.0 μg/ml (estimated) sample, alternating standard and sample. Incubate the plates for 16–18 hr at 37 C and measure the diameter of each circle of inhibition.

i) *Estimation of potency.*—Average the zone readings of the standard and average the zone readings of the sample of the three plates used. If the sample gives a larger average zone size than the average of the standard, add the difference between them to the 1.0 μg zone size of the standard curve. If the average sample value is lower than the standard value, subtract the difference between them from the 1.0 μg value on the curve. From the curve read the potencies corresponding to these corrected values of zone sizes.

Turbidimetric Method[26]

PROCEDURE

a) *Culture and inoculum.*—Employ the agar described in paragraph (*b*, 2), page 4, adjusted to a final pH 7.0 for maintaining the test organism, *Klebsiella pneumoniae* (PCI 602) noncapsulated. Transfer stock cultures every two weeks for test purposes. Transfer the organism to fresh agar slants and incubate at 37 C for 6 hr. Suspend the growth from two to three of these slants in sterile distilled water and add approximately 5 ml of culture suspension to each of two Roux bottles containing the same agar. Incubate the bottles for 6 hr at 37 C, harvest the growth and suspend in sufficient sterile distilled water to give a light transmission reading of 80 per cent, using a filter at 6500A in a photoelectric colorimeter. Keep the resulting suspension of organisms in the refrigerator and use for a period not to exceed 2 weeks. Prepare a daily inoculum by adding 6.0 ml of this suspension to each 100 ml of the nutrient broth (paragraph (*b*), p. 4) cooled to approximately 15 C.

b) *Working standard solutions.*—Add amounts of a 1000 μg/ml solution prepared from the stock solution (paragraph (*c*), p. 5) to nine 100 ml volumetric flasks containing sterile distilled water and bring to volume to give the working stock solutions listed in the tabulation. These nine flasks are well stoppered and maintained at approximately 15 C for 1 month. Prepare final dilutions daily by adding 2.1 ml of each of these nine working stock solutions to 4.8 ml of sterile distilled water. Add 1.0 ml of each final dilution to each of six 14 × 124 mm tubes (total 54 tubes). Add 9.0 ml of inoculated broth described above to each tube and place immediately in a 37 C water bath for 4 hr. The final concentration of streptomycin per ml of broth is also included in the tabulation.

[26] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

Amt. of Stand. Sol. (1,000 µg/ml), ml	Working Concent./ml (also % Concent.), µg	Final Concent. (µg/ml) after Addition of Dist. Water and Broth, µg
6.0	60	1.8
7.0	70	2.1
8.0	80	2.4
9.0	90	2.7
10.0	100	3.0
11.0	110	3.3
12.0	120	3.6
13.0	130	3.9
14.0	140	4.2

c) Preparation of sample.—Dilute the sample under test with sterile distilled water to contain 100 µg/ml (estimated). To 2.1 ml of the sample add 4.8 ml of sterile distilled water; add 1.0 ml of this dilution to each of six 14 × 124 mm tubes. Add 9.0 ml of the inoculated broth (paragraph (*a*)) to each tube and place immediately in a 37 C water bath for 4 hr. A control tube containing 1.0 ml of distilled water and 9.0 ml of the inoculated broth is similarly incubated. After incubation add 4 drops of formalin to each tube and read the light transmission in the photoelectric colorimeter, using a broad band filter having a wavelength of 5300 A.

d) Estimation of potency.—Average the light transmission readings for each concentration of the standard. Plot these values on cross-section paper, employing average light transmission readings as the ordinate and streptomycin concentration per ml of broth as the abscissa. Prepare the standard curve by connecting successive points with a straightedge. Since the final concentration of streptomycin per ml of broth is equivalent to the concentration per ml of the working stock solution (see tabulation), the latter concentrations for each concentration level of the standard may be expressed as percent and substituted on the abscissa of the standard curve. If this is done the percentage potency of the sample under test may be read directly from the standard curve.

Serial Dilution—K. Pneumoniae[n]

PROCEDURE

Dilute a 6 hr *Klebsiella pneumoniae* (ATCC 9997) culture grown in yeast beef broth, to 1×10^{-4} in a broth consisting of 0.75 per cent tryptone and water, pH 8.5. With a sterile automatic syringe dispense the diluted culture in 2 ml amounts in sterile, plugged test tubes, 13 × 100 mm. The filled tubes are placed in racks having two rows of holes, 10 holes per row. Each rack holds two tests.

Dilute the solution to be tested with sterile H_2O to give approximately 2 µg of streptomycin per ml. With sterile, acid-cleaned 0.2 ml Kahn pipets, add the following amounts of diluted streptomycin solution to a row of tubes containing diluted culture: 0.1, 0.088, 0.077, 0.068, 0.059,

[n] Donovick, R.; Hamre, D.; Kavanagh, F., and Rake, G.: Broth dilution method of assaying streptothricin and streptomycin, J. Bact. 50: 623, 1945.

0.052, 0.046, 0.040, 0.035 and 0.030 ml. Run one or more standard streptomycin solutions in triplicate with each day's tests. Incubate the tests at 37 C for 15–17 hr. Record the degree of growth in each tube after incubation. If (−) indicates no growth, (±) as a trace of growth and (+) as definite or strong growth, the (±) tube is considered the end-point. Calculate the activity of the unknown solution through use of the following equation:

$$\frac{Ax}{Ac} = \frac{Vc}{Vx}$$

where Ac = μg of streptomycin/ml in the standard solution; Ax = μg of streptomycin/ml in the unknown solution; Vc = volume of standard solution required to cause complete inhibition; Vx = volume of unknown solution required to cause complete inhibition.

NOTES

1. *Klebsiella pneumoniae* (ATCC 9997) is maintained in yeast beef broth consisting of beef extract 0.15 per cent, yeast extract 0.15 per cent, peptone 0.5 per cent, dextrose 0.1 per cent, dipotassium phosphate 0.368 per cent, potassium dihydrogen phosphate 0.132 per cent, sodium chloride 0.35 per cent (final pH 7.0). Incubate 6 hr at 37 C and store at about 4 C for use for 1 week or longer. A fresh 6 hr culture should give plate counts of 1 × 10^9 cells per ml. After storage the count drops gradually, and new subcultures should be made when the count drops to as low as 500 × 10^4 cells per ml.

2. *Klebsiella pneumoniae* (ATCC 9997) is sensitive to 0.04 μg of streptomycin per ml of the medium described in the procedure.

3. Various lots of tryptone interfere to varying degrees with the action of streptomycin. When new lots are to be used for the test, the sensitivity of the test organism to streptomycin should be tested in the new broth. To maintain a response to streptomycin consistent with previous results, the concentration of tryptone in the new test broth may be raised or lowered slightly (e.g., to 1.0 or 0.5 per cent tryptone), depending on whether the organism is more or less sensitive in the new broth.

4. The pH of the test medium is exceedingly important. Since the test broth is unbuffered, the pH, which has been adjusted with NaOH, will gradually drop on storage. Hence fresh broth is made every second day. Addition of phosphate buffers to the broth reduces the sensitivity of the test.

2. BODY FLUIDS

Plate Assay—Staph. Aureus[11]

PROCEDURE

Materials.—A strain of *Staphylococcus aureus* SM (PCI 1214) is used as the test organism. Maintain the culture in a uniform condition by daily transfer to F.D.A. nutrient broth at 37 C. The assay medium is a modified F.D.A. nutrient agar.

Preparation of plates.—Using a 6 hr culture of the test organism grown in F.D.A. broth, make serial tenfold dilutions through 10^{-4} in broth. Then make final dilution to 10^{-5} directly into the melted nutrient agar previously cooled to 45 C. With a calibrated wide-mouth pipet, add 10 ml of this melted seeded agar to each of a series of Petri dishes which are set aside until the agar solidifies. Warm beveled glass cylinders slightly by passage through a Bunsen flame and place immediately on the surface of the agar plate, thereby effecting a seal between the glass cylinder and the agar. Four such cylinders are placed on each agar plate. The glass cylinders are then filled with the properly diluted samples for assay and the plates incubated at 30 C for 16–18 hr. At the end of this period the diameters of the zones of inhibition are measured in millimeters.

Assay of blood, urine and tissue extracts.—When determining the concentration of streptomycin in blood, it is necessary to construct a standard curve of reference for each assay, using an aliquot of standard streptomycin. This is done by making dilutions of streptomycin containing 1, 2, 4, 8 and 16 μg/ml in serum obtained from each individual prior to treatment or from any normal donor. The diameters of the resulting zones of inhibition are plotted as ordinates (arithmetic scale) against the concentrations of streptomycin as abscissa (logarithmic scale).

Test serums containing unknown amounts of the drug are diluted with any normal human serum to contain approximately 5–15 μg/ml. This will insure a reading which will fall on the standard curve. Dilutions of the test sample as well as each level of the standard should be run in duplicate. The drug concentration in the serum sample is obtained by determining from the standard curve the streptomycin concentration corresponding to the diameter of the zone of inhibition and correcting for the dilution.

NOTES

1. Modified F.D.A. nutrient agar

Peptone (Siccum-Armour)	10.0 g
Bacto Beef Extract (Difco)	5.0 g
Salt (NaCl)	2.5 g
Agar-agar	10.0 g
Distilled water	1000.0 ml

Adjusted to pH 7.5–8.0 with N/1 NaOH.

[11] Stebbins, R. B., and Robinson, H. J.: Method for determination of streptomycin in body fluids. Proc. Soc. Exper. Biol. & Med. 59: 255, 1945.

2. The stainless steel cylinders described for official *Staph. aureus* plate assay for penicillin may be used (paragraph (*a*), p. 4).

3. In the assay procedure for urine, feces or tissue extracts, distilled water replaces serum as the diluent.

Serial Dilution in Body Fluids—B. Circulans[29]

PROCEDURE

Place 10 sterile Wassermann tubes in a rack. Add 0.5 ml of broth containing 1 per cent peptone, 0.5 per cent beef extract and 0.25 per cent NaCl (final pH 7.8–8.0) to all except the first tube. Prepare a similar series for each unknown solution. Prepare a standard solution containing 10 μg of streptomycin per ml and add 0.5 ml of it to each of the first two tubes of one series, mix the contents of tube 2 and transfer 0.5 ml to tube 3, etc., discarding 0.5 ml from tube 10. To another series add 0.5 ml of an unknown solution to each of the first two tubes and proceed as with the standard. Prepare an inoculum by making a 1 per cent suspension of a broth culture of *B. circulans* in the broth described above. Add 1.5 ml of inoculum to all tubes. Incubate at 37 C for 18–24 hr and examine for inhibition of growth. Determine the number of tubes of inhibition given by the standard and unknown solutions. If the 10 μg/ml standard solution gives seven tubes of inhibition, then seven tubes of inhibition in the unknown represents 10 μg of streptomycin per ml in the unknown, six tubes of inhibition represents 5 μg/ml, five tubes 2.5 μg/ml, etc., and eight tubes, 20 μg/ml in this twofold serial dilution technique.

NOTES

1. The number of tubes set up may be varied with expected concentrations in the unknown solution and/or the unknown may be diluted.

2. *Bacillus circulans* is a nonpathogenic spore former and is maintained in a nutritive medium consisting of peptone 0.5 per cent, yeast extract 0.15 per cent, beef extract 0.15 per cent, NaCl 0.35 per cent, glucose 0.1 per cent, dipotassium phosphate 0.308 per cent and potassium dihydrogen phosphate 0.132 per cent (final pH 7.0). Incubate at 37 C 24 hr and store in the refrigerator for use for a 2 month period.

3. *Bacillus circulans* is sensitive to 0.039 μg of streptomycin per ml of the medium described in the procedure. This strain may be procured from the Food and Drug Administration, Washington, D. C.

4. Proper care in preparation of media is essential. It is expedient to maintain a "reference" broth against which to check the sensitivity of the organism in subsequent batches of broth.

5. Chylous or hemolyzed serum and red blood cells interfere with the interpretation of this test.

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[29] Price, C. W.; Nielsen, J. K., and Welch, H.: Estimation of streptomycin in body fluids. Science 103: 56, Jan. 11, 1946.

2. BODY FLUIDS

Plate Assay—Staph. Aureus[29]

PROCEDURE

Materials.—A strain of *Staphylococcus aureus* SM (PCI 1214) is used as the test organism. Maintain the culture *in a uniform condition by* daily transfer to F.D.A. nutrient broth at 37 C. The assay medium is a modified F.D.A. nutrient agar.

Preparation of plates.—Using a 6 hr culture of the test organism grown in F.D.A. broth, make serial tenfold dilutions through 10^{-4} in broth. Then make final dilution to 10^{-5} directly into the melted nutrient agar previously cooled to 45 C. With a calibrated wide-mouth pipet, add 10 ml of this melted seeded agar to each of a series of Petri dishes which are set aside until the agar solidifies. Warm beveled glass cylinders slightly by passage through a Bunsen flame and place immediately on the surface of the agar plate, thereby effecting a seal between the glass cylinder and the agar. *Four such cylinders are placed on each agar plate.* The glass cylinders are then filled with the properly diluted samples for assay and the plates incubated at 30 C for 16–18 hr. At the end of this period the diameters of the zones of inhibition are measured in millimeters.

Assay of blood, urine and tissue extracts.—When determining the concentration of streptomycin in blood, it is necessary to construct a standard curve of reference for each assay, using an aliquot of standard streptomycin. This is done by making dilutions of streptomycin containing 1, 2, 4, 8 and 16 μg/ml in serum obtained from each individual prior to treatment or from any normal donor. The diameters of the resulting zones of inhibition are plotted as ordinates (arithmetic scale) against the concentrations of streptomycin as abscissa *(logarithmic scale).*

Test serums containing unknown amounts of the drug are diluted with any normal human serum to contain approximately 5–15 μg/ml. This will insure a reading which will fall on the standard curve. Dilutions of the test sample as well as each level of the standard should be run in duplicate. The drug concentration in the serum sample is obtained by determining from the standard curve the streptomycin concentration corresponding to the diameter of the zone of inhibition and correcting for the dilution.

NOTES

1. Modified F.D.A. nutrient agar

Peptone (Slocum-Armour)	*10.0 g*
Bacto Beef Extract (Difco)	5.0 g
Salt (NaCl)	2.5 g
Agar-agar	10.0 g
Distilled water	1000.0 ml

Adjusted to pH 7.5–8.0 with N/1 NaOH.

[29] Stebbins, R. B., and Robinson, H. J.: Method for determination of streptomycin in body fluids Proc. Soc. Exper. Biol. & Med. 59: 255, 1945.

NOTES

1. The standard streptomycin may be set up in serum or broth; the organism will show sensitivity to 0.5 μg/ml (complete inhibition in tube 5).

2. Fluids suspected of containing more than 50 μg of streptomycin per ml may be diluted with veal infusion broth accordingly in order to determine these higher values.

3. Approximately 150 human sera have been tested for inhibitory activity against the test organism (*Staph. aureus* SM), of which only one showed slight inhibitory activity.

4. Single veal infusion broth is used:

Double veal infusion	20 liters
Distilled water	20 liters
Paul Lewis peptone	400 g
Sodium chloride	200 g

(1) Warm double veal infusion to room temperature. (2) Filter through cotton and gauze—three layers. (3) Wash filter with cold distilled water. (4) Filter through double filter paper. (5) Bring up to volume with distilled water. (6) Weigh dry ingredients and add to the liquid. (7) Boil for a few minutes to dissolve. (8) Add 2N NaOH—430 ml. (9) Boil again. (10) Check pH (should be 8.0). (11) Put 8 liters into a 9 liter bottle, plug with cotton and cover with gauze and paper. (12) Sterilize at 15 lb pressure for 1 hr. (13) Store in the refrigerator. (14) When ready to use bring up to 8 liters. (15) Boil. (16) Adjust the pH to 7.4 with NaOH or HCl. (17) Filter through two layers of filter paper. (18) Dispense into flasks or tubes. (19) Sterilize in the autoclave.

Titration in Blood Serum—Klebsiella[31]

PROCEDURE

Culture medium.—Serum 1 part and distilled water 4 parts are boiled or steamed with 1 per cent glucose and enough of a saturated watery solution of phenol red to give a definite red color. For use, 1 ml of this medium is inoculated with 10 ml of a 24 hr broth culture of the test organism.

Test organism.—Friedländer's bacillus (Klebsiella 41) is very suitable, but if it is employed the patient's serum must be inactivated by heat before the test. (Staphylococci may be used.)

Make serial dilutions of the serum to be tested in normal saline solution, and to each dilution add an equal volume of the infected medium. Use 0.025 ml volumes and carry out the incubation in capillary tubes open at both ends and stuck flat on plasticine on a microscope slide.

[31] May, J. R.; Voureka, A. E., and Fleming, A.: Some problems in titration of streptomycin. Brit. M. J. 1: 627, May 10, 1947.

Serial Dilution in Body Fluids—Staph. Aureus[20]

PROCEDURE

Place 11 sterile Wassermann tubes in a rack. To the first four add consecutively the following amounts of standard streptomycin solution diluted in veal infusion broth containing 10 µg/ml, 0.5, 0.2, 0.15 and 0.1 ml. To the remaining tubes add consecutively the following amounts of standard streptomycin solution diluted in veal infusion broth containing 1.0 µg/ml, 0.5, 0.4, 0.3, 0.25, 0.2, 0.15 and 0.1 ml. Add the unknown fluid in the same amounts to a similar set of tubes. Add sufficient veal infusion broth (pH 7.4) to each tube to give a total of 0.5 ml per tube. From a 6 hr culture of *Staph. aureus*, strain SM, grown in the same veal infusion broth grown at 37 C, prepare a 1:1,000,000 dilution and add 0.5 ml to each tube in both series. Incubate at 37 C for 18–24 hr and determine the highest dilution showing complete inhibition in both the standard and the unknown. In the standard dilution series inhibition will occur at tube 5, which contains 0.5 µg. The *concentration of streptomycin per ml of* body fluid is shown in the tabulation.

Tube	Concentrated 1	2	3	4
Dilution	1:2	1:5	1:7.5	1:10
Ml of streptomycin stand. (10 µg/ml)	0.5	0.2	0.15	0.1
µg of streptomycin	*5.0*	*2.0*	*1.5*	*1.0*
Ml of veal infusion broth (pH 7.4)	..	0.3	0.35	0.4
Inoculum, ml	0.5	0.5	0.5	0.5
Value in µg/ml	1.0	2.5	3.75	5.0

Tube	1:10 Dilution 5	6	7	8	9	10	11
Dilution	1:20	1:25	1:33	1:40	1:50	1:75	1:100
Ml of streptomycin stand. (10 µg/ml)	0.5	0.4	0.3	0.25	0.2	0.15	0.1
µg of streptomycin	*0.5*	*0.4*	*0.3*	*0.25*	*0.2*	*0.15*	*0.1*
Ml of veal infusion broth (pH 7.4)	...	0.1	0.2	0.25	0.3	0.35	0.4
Inoculum, ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Value in µg/ml	10.0	12.5	16.5	20.0	25.0	37.5	50.0

[20] Romansky, M. J. (George Washington University School of Medicine and the Antibiotic Section Laboratory, Walter Reed General Hospital, Army Medical Center), with technical assistance of Bedsole, R.: *A method for assaying streptomycin in body fluids*, to be published.

ASSAY OF STREPTOMYCIN POTENCY: B. CHEMICAL METHODS

Colorimetric Method—Maltol[33]

PROCEDURE

Reagents.—These are 1N and 2N sodium hydroxide, 20 per cent sodium carbonate, 1 per cent ferric ammonium sulfate in 0.75N sulfuric acid, and phenol reagent (according to Folin and Ciocalteu).

Maltol formation.—To 5 ml of the streptomycin solution in a test tube add 1 ml of 2N NaOH. Immerse the tube in a boiling water bath for 3 min and cool for 3 min in cold water.

Color reactions.—Either ferric ammonium sulfate or the phenol reagent is used to develop a color with the maltol, depending on the amount of streptomycin originally present. Sensitivity with the ferric ammonium sulfate reagent is 500–2500 μg of streptomycin; with the phenol reagent, 20–250 μg.

Ferric ammonium sulfate reagent.—To the cooled alkaline solution add 4 ml of the ferric ammonium sulfate reagent. Shake vigorously and after 10 min determine the intensity of the purple color in a photoelectric colorimeter with filter 540. The light transmittance of the blank is nearly the same as for water and is constant, varying not more than ±0.5 deflection in the Evelyn photoelectric colorimeter.

Phenol reagent.—To the cooled alkaline solution add dropwise 1 ml of the phenol reagent and mix well. Let stand 1–2 min, add 3 ml of 20 per cent sodium carbonate and shake vigorously. Read after 10 min in a photoelectric colorimeter with filter 660. Prepare a blank simultaneously. Clinical preparations of streptomycin contain small quantities of material which react directly with the phenol reagent. Ordinarily this accounts for 2–6 per cent of the color value obtained after maltol formation. Therefore an additional determination is carried out with 5–10 times the quantity of the sample in exactly the manner described above except that the heating period with alkali is omitted. Since the formation of maltol from streptomycin occurs at an appreciable rate even at room temperature, the phenol reagent must be added *immediately* after the addition of the alkali.

Methanolic solutions of streptomycin.—If streptomycin is to be determined in methanolic solution, the ferric ammonium sulfate reagent is

[33] Boxer, G. E.; Jelinek, V. C., and Leghorn, P. M.: Colorimetric determination of streptomycin in clinical preparations, urine, and broth, J. Biol. Chem. 169: 153, 1947.

The test can be done in exactly the same way using larger volumes and incubating the mixtures in small test tubes. When serum water is used as the medium for titration and Klebsiella 41 or staphylococcus as the test organism the end-point comes at a dilution of 1:4,000,000–1:6,000,000. In blood about 1:1,000,000 concentration of streptomycin is required to inhibit growth completely, so that this method will indicate a smaller amount of streptomycin than the therapeutic level, whereas if broth were used this would not be so. Serum water is also more suitable for the test because it is an unbuffered medium and the end-point is more easily read than it is in broth.

Control.—Since the final end-point of the titrations depends on so many factors it is necessary to make a control titration of a known concentration of streptomycin (1:1,000,000) in human serum. A comparison of the end-point obtained with this and with the patient's serum will give an absolute measurement of the streptomycin content in the latter.

Chemical Assay of Body Fluids[14]

PROCEDURE A

Plasma reagents.—1. Dissolve 0.133 g of 4-[4-(p-chlorophenylazo-1-naphthyl)] semicarbazide in approximately 50 ml of redistilled methyl cellosolve by warming to 50 C. Dissolve 2.66 g of sodium acetate trihydrate in the solution so obtained, then add 8.3 ml of glacial acetic acid. Bring the mixture to room temperature and dilute with methyl cellosolve to 100 ml.

2. Chloroform, reagent grade.

3. Concentrated hydrochloric acid.

4. Trichloroacetic acid solution. Dissolve 15 g of trichloroacetic acid in sufficient water to yield 100 ml of solution.

Technique.—To 1 ml of plasma add 3 ml of water and 1 ml of trichloroacetic acid solution. After standing 20 min, centrifuge the mixture. Add 3 ml of the plasma centrifugate to 3 ml of the semicarbazide contained in a glass-stoppered centrifuge tube graduated at 3.5 ml. Heat the tube for 15 min in a boiling water bath and cool by immersion in ice water. Add 10 ml of chloroform and shake the stoppered tube at least 100 times. After separation of the two phases, remove the chloroform with a pipet, the tip of which has been drawn out to a fine capillary, or by means of a syringe and long needle. Repeat the extraction with two or more 10 ml quantities of chloroform. After removal of the last chloroform layer, add sufficient water to bring the aqueous phase to 3.5 ml. Treat 3 ml of this solution with 3 ml of concentrated hydrochloric acid and allow to come to room temperature. Determine the blue color intensity in a colorimeter equipped with a 580 mμ filter. Set the zero of the colorimeter with a mixture of equal volumes of concentrated hydrochloric acid and a solution obtained by adding 30 ml of methyl cellosolve to 100 ml of water.

PROCEDURE B

Urine reagents.—1. Sodium hydroxide, 2.5N.

2. Hydrochloric acid, 4N.

3. Chloroform, reagent grade.

4. Ferric nitrate solution. Dissolve 0.5 g of ferric nitrate nonahydrate in 100 ml of 0.035N nitric acid and dilute to 400 ml.

Technique.—Mix 3 ml of diluted urine (containing no more than 2 mg of streptomycin) with 0.7 ml of 2.5N sodium hydroxide in a 125 ml glass-stoppered pyrex bottle. Immerse this in a boiling water bath for 5 min, cool in ice water, add 0.5 ml of 4N hydrochloric acid and 60 ml of chloroform to the contents of the bottle and shake for 5 min. Withdraw 50 ml of the chloroform phase and shake for 5 min with 10 ml of the concentrated iron reagent. Withdraw a portion of the aqueous phase and determine the

[14] Marshall, E. K., Jr.; Blanchard, K. C., and Buhle, E. L.: Determination of streptomycin in plasma and urine, J. Pharmacol. & Exper. Therap. 90: 367, 1947.

applicable. Starting with 4 ml of methanol solution in a 10 ml graduated cylinder, add 2 ml of 1N NaOH and immerse the cylinders in a water bath at 65 C (± 1°) for 20 min. After cooling, add ferric ammonium sulfate reagent to the 10 ml mark. Intensity of the purple color is measured after 10 min.

Calibration.—Use a sample of standard streptomycin as the standard in the calibrations. Since the salts of streptomycin are hygroscopic and vigorous drying may cause some decomposition, two samples are weighed out at the same time; one is used for a moisture determination by drying for 3 hr at 100 C over phosphorus pentoxide in vacuo; the other is used for the calibration and a correction is made for the water content.

NOTES

1. The phenol reagent is prepared as described by Folin, O., and Ciocalteau, V.: J. Biol. Chem. 73: 627, 1927.

2. The determination of streptomycin in broth and urine requiring additional procedures is given in the original publication.

Colorimetric Method—Oxidized Nitroprusside[11]

PROCEDURE

Place 1.0 ml of a solution containing approximately 0.1–0.3 mg of streptomycin in a test tube and add 1.0 ml of freshly prepared oxidized nitroprusside reagent and 3.0 ml of distilled water. Allow to stand at room temperature for 10 min and read the light transmission in a photoelectric colorimeter employing a *540 mμ broad band filter*. *Prepare* a standard curve employing 0.1, 0.15, 0.20, 0.25 and 0.30 mg/ml of streptomycin standard. Determine the amount of streptomycin in the unknown solution by locating the point on the standard curve corresponding to the transmission percentage.

NOTES

1. Prepare the oxidized nitroprusside reagent (Weber's modification) as follows:

10% sodium nitroprusside	1 ml
10% potassium ferricyanide	1 ml
10% sodium hydroxide	1 ml
Distilled water	9 ml

Allow to stand 30 min before use.

2. The test constitutes a general reaction for guanidines.

[11] Sullivan, M. X., and Hilmer, P. E.: Chemical studies of streptomycin, Am. Chem. Soc., Abst. of 109th Convention, Div. Biol. Chem., p. 4B, April, 1946.

ASSAY OF TYROTHRICIN POTENCY: BIOLOGIC METHOD[11]

PROCEDURE

a) *Stock standard.*—A solution containing 1 mg/ml of a mixture consisting of 20 per cent gramicidin and 80 per cent tyrocidine in 95 per cent ethyl alcohol is the stock standard. Maintain in the refrigerator at 15 C and take precautions to prevent evaporation.

b) *Working standard.*—Dilute the stock standard so that 10 μg/ml of tyrothricin is obtained, using 95 per cent ethyl alcohol. Further dilute to 1 μg/ml, using a solution of the following composition:

Propylene glycol	43.7 ml
95% ethyl alcohol	12.5 ml
Distilled water, q.s.	100.0 ml

c) *Organism.*—*Streptococcus faecalis*, group D, nonpathogenic (PCI 1305).

d) *Media.*

1) Nutrient agar for carrying test organism.

Beef extract	0.5%
Peptone	1.0%
NaCl	0.5%
Agar	1.5%
H_2O	1000.0 ml

Adjust to pH 7.0 before sterilization.

2) Assay and daily transfer broth.

Tryptic digest of lactalbumin	8.0 g
Distilled water	1000 0 ml

or

Pancreatic digest of casein	8.0 g
Distilled water	1000.0 ml

e) *Technique of test.*—Further serially dilute the working standard with diluting solution (paragraph (*b*)) to obtain concentrations of 0.5, 0.25, 0.125, 0.0625 μg/ml of tyrothricin and designate as B, C, D, E, respectively. The dilution designated A is the working standard (1 μg/ml). Prepare 5 ml of each solution. The corresponding values for gramicidin in the respective dilutions are:

[11] Food and Drug Administration, Federal Security Agency, Washington, D. C. Unpublished data.

relative optical density in a colorimeter equipped with a standard green filter (545 mμ). The absorption peak of the iron-maltol complex is broad and reaches a maximum at 530 mμ. Correct the colorimeter reading for the blank on each sample of urine used. This is determined by treating another sample of the urine in the fashion already described but without addition of sodium hydroxide and heating.

stock standard to 1 ml of the sample. Dilute the mixture 1:500, first by diluting 1:50 with alcohol and then 1:10 with the diluting fluid to obtain 1 μg/ml of added tyrothricin. Subsequent serial dilutions of the 1 μg/ml solution are carried out and the seeded broth is added as in the routine method. The resulting end-point is tube 7 of the series if the end-point for the standard series occurs at tube 5. Thus, growth is inhibited by a dilution of the mixture which is twice as great as the standard. The test sample therefore contains an amount of tyrothricin equal to the standard.

Tube	A	B	C	D	E
Gramicidin value (µg/ml)	.2	.1	.05	.025	.0125

From each of the tubes A, B, C, D and E transfer 0.15 and 0.1 ml to the bottom of chemically clean, sterile, dry test tubes (20 × 150 mm). Add a control tube containing 0.15 ml of the diluting solution. Run standard in triplicate. The following values represent the amount of gramicidin in the respective dilutions of the standard:

Tube	1	2	3	4	5	6	7	8	9	10	C
Gramicidin	.03	.02	.015	.01	.0075	.005	.00375	.0025	.001875	.00125	0

Dilute the sample to be assayed with 95 per cent ethyl alcohol to contain 10 µg of tyrothricin per ml (estimated). Dilute to a working solution containing 1 µg of tyrothricin per ml with the diluting solution (paragraph (b)). Further serially dilute the 1 µg/ml solution with the diluting solution in the same manner as was done with the working standard, so as to give estimated gramicidin concentrations of the assay sample which correspond to the respective concentrations of the standard. Prepare solutions of the unknown sample to be assayed in triplicate.

Seed the assay broth from a 24 hr broth culture of the test organism to give a 1 per cent suspension. Shake the seeded broth thoroughly and add 5 ml to each tube of the triplicate series of the unknown sample and to each tube of the standard series. Shake all tubes vigorously and place in a 37 C water bath for 24 hr.

f) Interpretation.—The test should give a sharp end-point. The end-point of the standard should be tube 5 or 6 of the dilution series representing 0.0075 or 0.005 µg of gramicidin. Thus, in an assay, if the end-point for the standard is tube 5 and tube 7 is the end-point of the unknown sample, the unknown sample contains 0.0075/0.00375, which represents twice the amount of tyrothricin in the working solution of the unknown as in the working standard, or 2 mg/ml in the undiluted sample.

Supplementary Method

It has been found that with the foregoing method certain preparations of tyrothricin, because of interfering substances, yield results which indicate low values of tyrothricin. Therefore samples that show a deficiency of tyrothricin should be check assayed as follows:

PROCEDURE

Add to the sample an equal volume of the standard containing an amount of tyrothricin equivalent to that in the sample. Dilute with alcohol so that each milliliter of the mixture contains 10 µg of the added tyrothricin standard. Further dilute the mixture with the diluting fluid (paragraph (b)) to obtain 1 µg/ml on the basis of added tyrothricin and proceed as in the routine method.

Thus, if the sample claims 1000 µg of tyrothricin per ml, add 1 ml of

hr at 37 C. Suspend the growth in 10 ml sterile NaCl. Determine density of the suspension by measuring the light transmission of a 1:50 dilution in a colorimeter using a filter of 0500 A wavelength. When the transmission is 75 per cent a 0.5 per cent inoculum is required. Vary the percentage of inoculum with the density of the suspension. The same suspension may be used for 2 weeks if maintained at refrigerator temperature.

3. Standard aqueous solutions of bacitracin may be used for 1 month if kept under refrigeration.

Turbidimetric Method[77]

PROCEDURE

Place in a rack six tubes (15 × 130 mm) for *each level* of the standard and six tubes for each unknown to be run. Dilute a standard bacitracin preparation to contain 0.1, 0.141, 0.200, 0.282, 0.398 and 0.562 unit* per ml. Dilute the sample under test to contain 0.25 units/ml (estimated). Add 1 ml amounts of each of these concentrations to the appropriate tubes. Add 9.0 ml of refrigerated broth inoculated with (ca.) 6.0 ml/100 ml of a standardized culture of *Staphylococcus aureus* (PCI 1203) to each tube. Place all tubes immediately in a 37 C water bath. After 4 hr of incubation add 4 drops of formalin to each tube and read turbidity in a photoelectric colorimeter, using a broad band filter of 5300 A wavelength. Average the colorimeter readings at each standard level. Plot these averages on semilog graph paper, employing units per tube as the abscissa and light transmission as the ordinate. Connect each point with a straightedge and read the value of the unknown from the curve in units per tube. Units per tube times 400 will give the percentage potency of the sample.

NOTES

1. Maintain *Staph. aureus* (PCI 1203) on a nutritive medium consisting of peptone 5.0 g, beef extract 3.0 g, agar 15.0 g, distilled water to make 1000 ml, final pH 7.0. Transfer every 2 weeks. Nutrient broth is inoculated on the day of the test from a stock slant, and the organism allowed to grow until light transmission, employing a 5300 A filter, measures 75 per cent. Broth cultures may be held at refrigerator temperature overnight.

2. The nutrient broth is peptone 5.0 g, yeast extract 1.5 g, beef extract 1.5 g, sodium chloride 3.5 g, glucose 1.0 g, dipotassium phosphate 3.68 g, potassium dihydrogen phosphate 1.32 g, distilled water to make 1000 ml (final pH 7.0).

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[77] Food and Drug Administration, Federal Security Agency, Washington, D. C. Unpublished data.

* The present bacitracin unit is defined as "that amount which, when diluted 1:1024 in a series of twofold dilutions in 2 ml of beef infusion broth, completely inhibits the growth of a stock strain of a group A hemolytic streptococcus, when the inoculum used to seed the tubes is 0.1 ml of a 10^{-3} dilution of an overnight culture in blood broth."

ASSAY OF BACITRACIN POTENCY: BIOLOGIC METHODS

Plate Assay[14]

PROCEDURE

Use three Petri dishes for each point to be determined on the standard curve and three for each unknown solution to be assayed. Place 21 ml of base agar, containing 0.15 per cent Bacto Beef Extract, 0.3 per cent Bacto Yeast Extract and 0.6 per cent Bacto Peptone and 1.5 per cent Bacto Agar (final pH 6.3–6.8) in sterile Petri dishes and allow to solidify. Prepare a seed agar consisting of 0.15 per cent Bacto Beef Extract, 0.3 per cent Bacto Yeast Extract, 0.4 per cent Bacto Casitone, 0.6 per cent Bacto Peptone, 0.1 per cent Bacto Dextrose and 1.5 per cent Bacto Agar. Cool to 50 C and inoculate with the calculated amount of *Micrococcus flavus* suspension, then add 4 ml of this seed layer to each base plate.

Prepare aqueous solutions of standard bacitracin to contain 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6 and 2.0 units/ml. Use the 1.0 unit/ml solution as a reference point on all plates. Dilute unknown solutions to 1.0 unit/ml (estimated). Place six standard steel cups equidistant on each plate. Pipet into alternate cups the 1.0 unit solution (reference point) and into remaining cups the appropriate standard or unknown solutions, using three plates each. Incubate the plates at 37 C for 18 hr. Measure the zone of inhibition to 0.1 mm. Average the 63 (nine for each of seven points) 1.0 unit zones of inhibition from the curve to obtain the average reading for the reference point. Average the nine readings for each point on the curve and, using the observed variation of the 1.0 unit reference point average, correct the average value obtained for each point. Plot these corrected values on two cycle semilog paper and draw the standard curve through these points. Estimate potencies from the curve.

NOTES

1. Maintain stock cultures of *M. flavus* on slants containing 0.3 per cent Bacto Beef Extract, 0.3 per cent Bacto Peptone and 1.5 per cent Bacto Agar. Incubate at 37 C for 18 hr; maintain the cultures in the refrigerator.

2. Prepare an inoculum of *M. flavus* by seeding a Roux bottle containing agar (described in note (1)) with organism from a slant. Incubate 18

[14] Bennett, R. E., Commercial Solvents Corporation, Terre Haute, Ind. Unpublished data.

rise in temperature, or if the sum of the temperature rises of the three animals exceeds 1.4 C, repeat the test on five additional animals. The sample is nonpyrogenic if not more than one of these five animals shows a rise in temperature of 0.6 C or more above the control temperature of such animal.

The same test is utilized for *streptomycin* with a test dose of 1.0 ml/kg of a solution containing 10 mg/ml.

TOXICITY: SAFETY TESTS FOR PENICILLIN AND STREPTOMYCIN[39]

PROCEDURE

In each of five mice, within the weight range of 18–25 g, inject intravenously 0.5 ml of a solution of the penicillin sample prepared by diluting with sterile distilled water to approximately 4000 units/ml. The injection should be made over a period of not more than 5 sec. If no animal dies within 48 hr, the sample is nontoxic. If one or more animals die within 48 hr, repeat the test with five unused mice weighing 20 g ($\pm$0.5 g) each; if all animals survive the repeat test, the sample is nontoxic.

The same test is utilized for *streptomycin* with a test dose of 0.5 ml of a solution containing 2 mg/ml.

STERILITY OF SAMPLE[40]

PROCEDURE

a) *Culture medium.*—Use U.S.P. fluid thioglycollate medium I or a dehydrated mixture which, when reconstituted with distilled water, has the same composition as such medium and has growth-promoting, buffering and oxygen tension-controlling properties equal to or better than those of such medium.

In preparation of the medium from either the individual ingredients or any dehydrated mixture, avoid contamination with calcium.

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[39] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.
[40] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

PYROGEN TESTS FOR PENICILLIN AND STREPTOMYCIN[18]

PROCEDURE

a) Test animal.—Use healthy rabbits weighing 1500 g or more which have been maintained for at least 1 week on a uniform, unrestricted diet and have not lost weight during this period. For subsequent tests, animals utilized for previous tests may be used after a rest period of not less than 2 days. Use a clinical rectal thermometer after it has been tested in a rabbit to determine the time required to reach maximal temperature. (Other recording devices of equal sensitivity are acceptable.) Insert the thermometer or other recording device beyond the internal sphincter and allow it to remain long enough to reach maximal temperature as determined above. Make four rectal temperature readings on each of the animals to be used in the test at 2 hr intervals, 1–3 days before such use (this may be omitted for any animal used in such tests during a preceding period of 2 weeks). House the test animals in individual cages and protect them from disturbances likely to cause excitement. Exercise particular care to avoid exciting the animals on the day of taking the control temperatures and on the test day. Maintain the animals in an environment of uniform temperature (±5 F) at all times.

b) Conduct of test.—Heat all syringes and needles to be used in a muffle furnace at 250 C for not less than 30 min to render them pyrogen-free and sterile. Perform the test in a room held at the same temperature as that in which the animals are housed. During the test restrain the animals in individual stocks. Withhold all food from 1 hr before the first temperature reading until after the final reading of the day. Take a control temperature reading not more than 15 min after the animal is removed from the cage. Use three animals for each test, but do not use those with control temperatures of 38.8 C or under and 39.9 C or over. Dilute the penicillin sample with pyrogen-free, sterile distilled water to a concentration of 2000 units/ml and warm to approximately 37 C. Inject 2000 units (estimated) per kg of rabbit intravenously through an ear vein within 15 min after the control temperature reading. Read temperatures 1 hr after injection and each hour thereafter until three readings have been made. The sample is nonpyrogenic if, when so tested, no animal shows a rise in any of the temperature readings, after injection, of 0.6 C or more above the control temperature of such animal. If only one animal shows such a

[18] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

pressure caused by 0.1 μg/kg of histamine base (not less than 20 mm Hg) is subsequently employed as the standard in testing samples.

Inject 3 mg/kg of the sample of streptomycin which has been diluted in saline to contain 3.0 mg (estimated) of streptomycin per ml, maintaining the 5 min injection schedule. If a significant drop is encountered, the dose is repeated after the animal has been retested with the standard histamine. If the blood pressure remains reasonably stable, six to eight samples may be examined. The product is satisfactory if the fall in blood pressure obtained with 3 mg of streptomycin per kg of body weight is no greater than the fall obtained with 0.1 μg of histamine base per kg of body weight. (Dogs may be substituted for cats in this test provided the ratio of the doses of streptomycin and histamine employed is the same.)

b) Penicillin preparation.—Dissolve the sample to be tested in sufficient sterile, freshly prepared solution of 1:300 hydroxylamine hydrochloride, adjusted to pH 6.0 with sodium hydroxide, so that each milliliter contains 5000–10,000 units. Shake vigorously. Let stand 1 hr and transfer 1 ml aseptically to each of four tubes containing 15 ml of fluid thioglycollate medium. Inoculate one of these tubes with 1.0 ml of a 1:1000 dilution of an 18–24 hr broth culture of *Staphylococcus aureus* (FDA 209-P) and incubate all four tubes for 4 days at 37 C. The inoculated tube should show growth at the end of 4 days; if it does and no other tube shows growth, the sample is sterile.

c) Streptomycin preparation.—Add aseptically 20 ml of sterile distilled water to approximately 50 mg of streptomycin. Transfer the equivalent of 25 mg of this solution to 5 ml of a sterile solution of 1:300 hydroxylamine hydrochloride adjusted to pH 6.0 with sodium hydroxide. The hydroxylamine hydrochloride is sterilized at 15 lb pressure (121 C) for 20 min and prepared once a week. Mix thoroughly and let stand for 1 hr. Transfer 1.0 ml of the inactivated streptomycin to each of four tubes containing 15 ml of fluid thioglycollate medium. Inoculate one of these tubes with 1.0 ml of a 1:1000 dilution of a 3–4 hr broth culture of *Klebsiella pneumoniae* (PCI 602) and incubate all four tubes for 4 days at 37 C. The inoculated tube should show growth at the end of 4 days; if it does and no other tube shows growth, the sample is sterile.

HISTAMINE CONTENT (STREPTOMYCIN)[41]

PROCEDURE

Use a healthy adult cat as the test animal. Determine weight and place under general anesthesia by administering sufficient (150 mg/kg) sodium phenobarbital intraperitoneally. Surgically expose the right carotid, separating it completely from all surrounding structures, including the vagus nerve, by blunt dissection, and cannulate. Surgically expose the femoral vein. Start the recording kymograph and inspect the tracings for amplitude of excursion and relative stability of pressure. Determine the sensitivity of the animal by injecting into the femoral vein standard solutions of histamine made to contain the equivalent of 1.0 μg of histamine base per ml. Make injections at not less than 5 min intervals, using doses of 0.05, 0.1 and 0.15 μg of histamine base per kg. Repeat these injections, disregarding the first series of readings, until the drop caused by equivalent doses of histamine is relatively uniform. The fall in blood

[41] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

pressure caused by 0.1 μg/kg of histamine base (not less than 20 mm Hg) is subsequently employed as the standard in testing samples.

Inject 3 mg/kg of the sample of streptomycin which has been diluted in saline to contain 3.0 mg (estimated) of streptomycin per ml, maintaining the 5 min injection schedule. If a significant drop is encountered, the dose is repeated after the animal has been retested with the standard histamine. If the blood pressure remains reasonably stable, six to eight samples may be examined. The product is satisfactory if the fall in blood pressure obtained with 3 mg of streptomycin per kg of body weight is no greater than the fall obtained with 0.1 μg of histamine base per kg of body weight. (Dogs may be substituted for cats in this test provided the ratio of the doses of streptomycin and histamine employed is the same.)

SECTION II

Circulation—Blood Flow Measurement

ASSOCIATE EDITOR—*Harold D. Green*

INTRODUCTION

THE ASSIGNMENT for this year's physiology section is Circulation. Because of limitations of space, recent considerable interest in blood flow and the fact that several new techniques have appeared for its measurement, the section is limited almost entirely to a presentation of blood flow, including cardiac output (total flow), flow in various regions of the body and an interpretation of the measurements in terms of vasomotor activity. Techniques for pressure measurement were recently reviewed by the editor.*

Blood flow measurements have been used: (*a*) for physiologic studies of regulation of the circulation in response to fluctuating demands in various regions, to changes in posture and acceleration and in the maintenance of body temperature; (*b*) in study of disturbances of circulation, such as arteriosclerosis, Buerger's disease, hypertension, A-V fistula and congenital cardiac defects, and (*c*) in the pharmacologic study of drugs and synthetic agents designed to control hypertension, relieve vascular engorgement and improve peripheral circulation.

Recent developments have emphasized methods applicable to man and unanesthetized animals. In acute experiments, flow measurements in the opened blood vessel have the advantage over most other methods of giving direct and fairly accurate quantitative readings, but require anesthesia and some form of anticoagulant. Both venous outflow and arterial inflow methods are applicable. For the former, measurements of

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* Green, H. D.: Circulation: Physical Principles, in Glasser, O. (ed.): *Medical Physics* (Chicago: Year Book Publishers, Inc., 1944), pp. 208 ff.

venous drainage or of the mean flow in veins returning blood to the heart are suitable; for the latter, the meter must either record the instantaneous flow or be able to integrate correctly the pulsatile flow found in arteries, or else the pulsatile flow must be converted to a steady flow.

Blood flow in the whole body is determined from the cardiac output measured by the Fick method and other gasometric techniques (p. 224) and by dye injection (p. 221) and in a qualitative sense by the ballistocardiograph (p. 221) and pulse wave velocity registration (p. 221). Cutaneous blood flow can be estimated by the photoelectric (p. 177), the bow string (p. 166), and the Hewlett-Van Zwaluwenburg plethysmographic techniques (see p. 182), by registration of cutaneous temperature or heat radiation (see p. 140) and in experimental animals by direct measurement of inflow or outflow (see pp. 68–130) in a vessel supplying principally a cutaneous area. Blood flow in muscles may be determined by measurements of deep muscle temperatures (p. 146), by plethysmography (p. 182) and by direct blood flow measurement in vessels supplying primarily muscular tissue. Hepatic (p. 190), cerebral (p. 204), coronary and renal blood flows (p. 191) have been studied by chemical techniques and the last three also by direct blood flow measurements. Flow in blood vessels may be measured in both the opened (pp. 78–89, 96–108) and unopened vessel (pp. 89 and 108).

—Harold D. Green.

VENOUS DRAINAGE RECORDERS

HAROLD D. GREEN

ONE OF THE SIMPLEST and in many respects the most satisfactory method for recording flow through an organ or portion of the body of experimental animals is to collect the outflow from the cannulated vein in a suitable chamber and to measure or record the volume periodically.

I. Slope or Integrating Recorders

a) *Volume recorders.*—The rate of accumulation of venous outflow blood may be indicated mechanically by causing the blood to enter the bottom of a closed chamber and recording the volume of displaced air with a bellows, bell, float or piston recorder as a sloping line on a kymograph record (Fig. 1). A sensitive recorder to measure very small flows could make use of the almost frictionless movement which can be obtained by use of a dry syringe in which either the barrel or the plunger is continuously revolved (3).

b) *Pressure registration.*—The rate of accumulation of venous outflow blood can be indicated, as proposed by Ligon (8), by recording on a photokymograph by means of an optically recording pressure manometer the increase of pressure in the blood in the chamber as the fluid level rises.

c) *Mechanically indicating weight recorder.*—When a sensitive volume recorder is not available and when mechanical indicating is desired, a highly satisfactory recorder may be constructed from a triple beam balance, sensitive to 0.1 g (Fig. 2). The weight of the chamber is counterbalanced and the weight of the blood opposed by a sensitive spring. A sensitivity of 0.5 ml/mm deflection is obtained by using a long recording arm; the arc is negligible so that the record can be handled as if written by a vertically moving pointer. I attempted to use a pulley and counterweight in place of the balance to obtain vertical recording, but friction in the bearing was such that the pointer would not respond to an increment of less than 5 ml. The balance type of recorder can be adapted to optical recording by use of mirrors (Fig. 3).

d) *Mechanisms for emptying outflow chamber.*—To facilitate use of such recorders an arrangement is usually provided for emptying the measuring chamber periodically. This may be accomplished by manual operation of

stopcocks or pinch clamps. More frequently an automatically operating siphon (Fig. 2) is used (7). It is simple to construct and is the only mechanism suitable for use with the balance meter (Fig. 2). However, at low rates of flow the siphon may not start unless made of small bore tubing, i.e., less than 6 mm internal bore. On the other hand, such small bore will not accommodate large rates of flow without causing considerable interruption during which measurement of flow is not obtained, and at high rates of flow it tends to empty before the chamber is full.

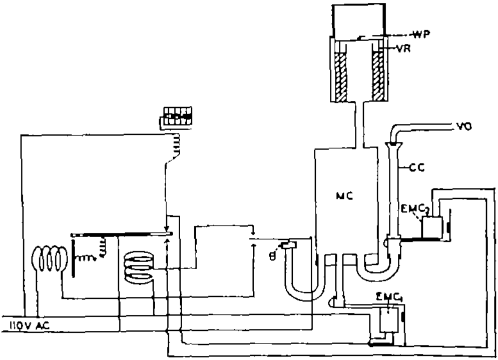

FIG. 1.—Arrangement for respirometer type of venous outflow recorder, designed to empty itself automatically. *WP*, writing pointer; *VR*, volume recorder; *VO*, venous outflow cannula; *CC*, collection chamber; *MC*, measuring chamber; *EMC*$_1$, *EMC*$_2$, electric clamps to control flow from and into measuring chamber; *B*, sensitive bellows and contact for signaling height of blood in measuring chamber. Apparatus at left is a latching relay activated by bellows *B* which opens *EMC*$_1$ and closes *EMC*$_2$ when blood in measuring chamber has reached proper height, and closes *EMC*$_1$ and opens *EMC*$_2$ when blood has dropped to proper point. Device above left is a counter for totalizing stroke of the flow meter.

Emptying may be by electrically operated clamps controlled by a latching relay operated by a bellows and contact, the latter controlled by the blood level in the measuring chamber (Fig. 1). Since an electrical contact is made at each end of the stroke, this arrangement can be used to totalize the flow over a considerable length of time by connecting an impulse counter in parallel with one of the magnets (Fig. 1).

c) Calculation of flow.—With any of the above described outflow meters the slope of the recorded line is readily converted to ml/min by construct-

ing a protractor from transparent plastic sheeting which can be applied to the curve and which will convert the slope directly to rate of flow. Such a device can be used only where the piston recorder rises vertically and linearly with inflow of blood and where the drum speed remains constant. Calculations for such protractor are

$$F = \theta \times \frac{R}{L}$$

where F is rate of flow in ml/min; θ, measured angle between slope line

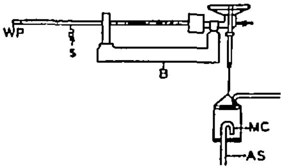

Fig. 2.—Device for recording venous outflow. Outflow enters measuring chamber MC through outflow cannula. Weight of chamber is supported on sensitive balance B, which is just adjusted to support the chamber. Sensitive spring S pulls writing pointer WP to lower position when measuring chamber is empty. As chamber fills, pointer moves up until automatic siphon AS causes chamber to empty. Pointer may extend 30–50 cm from axis of the balance.

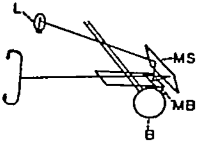

Fig. 3.—Mirror arrangement for attachment to the balance (Fig. 2, B) to convert it to optical recording. L, projection lamp; MB, mirror mounted on balance over fulcrum; MS, mirror for reflecting light from lamp down on mirror MB, then directing reflected light from MB into photokymograph shown in lower left-hand corner.

and base line: tan θ, the tangent of this angle—from trigonometric tables; R, calibration of the flow meter in ml/mm rise of the recording line; and L, calibration of the kymograph drum in min/mm movement of the paper.

When the emptying of the chamber is sufficiently accurate, the rate of flow/min can be calculated from the length of the cycle in seconds:

$$F = \frac{V \times 60}{T}$$

where F is flow in ml/min; V, stroke volume of chamber, and T, length of stroke in seconds.

II. Direct Reading Rate of Flow Meter

The outflow meter may be modified (Fig. 4) so that the cycle has a constant length by rotating an L stopcock by means of a magnet controlled by a relay operated from the laboratory time signal system.

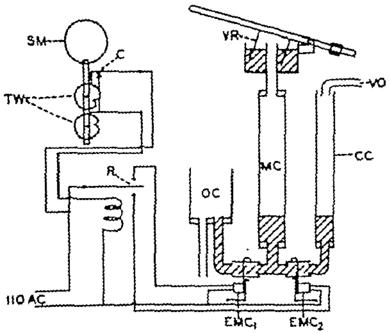

FIG. 4A.—Arrangement for use as continuous recording outflow meter. *VR*, volume recorder; *VO*, venous outflow cannula; *CC*, collection chamber; *MC*, measuring chamber; *OC*, overflow chamber which assures that blood in measuring chamber drops to constant level during each outflow stroke of meter; EMC_1, EMC_2, electric clamps for allowing blood to flow, respectively, from and into measuring chamber from collection chamber; *SM*, synchronous motor; *TW*, timing wheels from laboratory timing system so arranged that they close contact *C* for approximately 2 sec and leave it open approximately 8 sec, total cycle being 10 sec; *R*, relay operated by timing system.

With this arrangement the rate of flow during each 10 sec interval is signaled in terms of height of rise of the volume recorder line. This is an extremely convenient procedure, particularly for study of the response of perfused tissues, in that the rate of flow is continuously and immediately available without resort to calculation and in that the kymograph drum may be run much slower, thus conserving paper. With this arrangement it is immediately apparent when flow has returned to control rates and thus when another injection may be made (Fig. 5, p. 73). The first meter of this type was described by Gaddum (5).

ing a protractor from transparent plastic sheeting which can be applied to the curve and which will convert the slope directly to rate of flow. Such a device can be used only where the piston recorder rises vertically and linearly with inflow of blood and where the drum speed remains constant. Calculations for such protractor are

$$F = t \times \frac{R}{L}$$

where F is rate of flow in ml/min; θ, measured angle between slope line

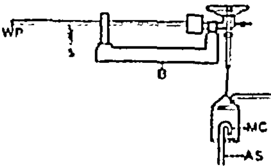

Fig. 2.—Device for recording venous outflow. Outflow enters measuring chamber MC through outflow cannula. Weight of chamber is supported on sensitive balance B, which is just adjusted to support the chamber. Sensitive spring S pulls writing pointer WP to lower position when measuring chamber is empty. As chamber fills, pointer moves up until automatic siphon AS causes chamber to empty. Pointer may extend 30–60 cm from axis of the balance.

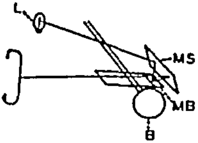

Fig. 3.—Mirror arrangement for attachment to the balance (Fig. 2, B) to convert it to optical recording. L, projection lamp; MB, mirror mounted on balance over fulcrum; MS, mirror for reflecting light from lamp down on mirror MB, then directing reflected light from MB into photokymograph shown in lower left-hand corner.

and base line: tan θ, the tangent of this angle—from trigonometric tables; R, calibration of the flow meter in ml/mm rise of the recording line; and L, calibration of the kymograph drum in min/mm movement of the paper.

When the emptying of the chamber is sufficiently accurate, the rate of flow/min can be calculated from the length of the cycle in seconds:

$$F = \frac{V \times 60}{T}$$

operation of a signal magnet by contact of the fluid with the electrodes is not possible. To overcome this, amplifiers, sensitive relays and other devices have been interposed between contacts and signal magnet (2, 6, 11, 13).

To simplify analysis of such records and to give a continuous picture of the rate of flow, Meiners (10) devised an apparatus in which a writing pointer moves with a constant speed in the ordinate direction, returning

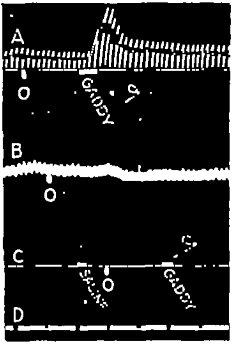

FIG. 5.—Sample record obtained with flow meter described in Figure 4B. *A*, record written by volume recorder; *B*, mean arterial pressure; *C*, signal for injection of saline and injection of vasodilator (Gaddy, plain, nonfiltered, 0.4 ml). Response to saline is the small rise in stroke at left edge of record in curve *A*; response to vasodilator is larger series of rises of volume recorder just above the figure 9. *D*, time, each space being 1 min. Calibration was such that maximal flow during vasodilation was approximately 10 ml/10 sec, and approximately linear. Calibration should be made while meter is making strokes, i.e., with constant flow, rather than by measuring deflection in response to addition of a single volume of fluid.

abruptly to its starting position each time a drop hits the contact. The time between drops is thus indicated by height of the ordinate, faster flows giving shorter lines and slower flows longer lines.

The position of the contacts has been modified to record inflow. This is accomplished by causing a constant flow of perfusion fluid from a Mariotte flask into a perfusion reservoir at a rate which is faster than the perfusion. The excess, escaping through an overflow spout, drips on a pair of contacts arranged to record as above. Rapid inflow is thus signaled by a slower rate of dropping, and slower inflow by a faster rate of dropping.

Vertical movement of the pointer is accomplished by a constant speed

In all of these types of meters the venous outflow must be from a tube into a collection chamber, as shown in the illustrations, rather than directly into the bottom of the measuring chamber. The latter would introduce periodic variations in resistance to outflow which would interfere with accurate measurements.

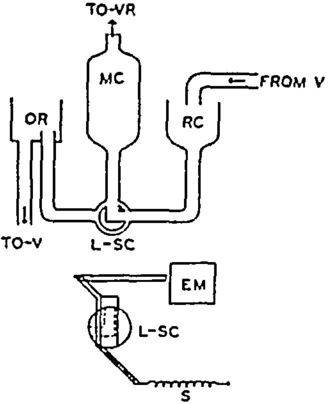

FIG. 4B.—Modification of Figure 4A, using L stopcock. This is more satisfactory than electromagnetic clamps because it avoids movement of volume recorder caused by compression of rubber tubes by clamps EMC_1 and EMC_2 (Fig. 4A). *RC*, receiving chamber into which blood flows from vein; *L–SC*, L stopcock; *MC*, measuring chamber; *TO–VR*, connection to volume recorder; *OR*, outflow reservoir; *TO–V*, outflow to device arranged to return blood to animal's vein; *EM*, electromagnet (conveniently a solenoid) which rotates stopcock to right; *S*, spring which returns stopcock to position shown in lower half of drawing. Electromagnet *EM* is operated by connecting it to appropriate contact on relay shown in Figure 4A; i.e., connection should be same as for EMC_1. The solenoids are those used in operating clutch, valves, etc., on the Bendix washing machine and are obtainable from Bendix service departments.

III. Drop Recorders

For very low rates of flow, drop recorders can be used to measure either inflow or outflow. Accuracy depends on the drop size remaining the same at all rates of flow. Most of the electrical circuits devised to allow registration and counting of the drops are arranged so that the drop shorts a pair of contacts, causing a signal magnet to record the time of the drop on a kymograph. Owing to the high electrical resistance of many fluids and their tendency to cause a coagulum to form on the contacts, direct

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V. Strain Gauge

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To replace rather cumbersome and frequently inaccurate mechanical devices, we have introduced a strain gauge to obtain a continuous record of increase in weight of the collecting vessel. It is a small compact unit which may be placed directly under the receiving vessel and connected to the recording system by light flexible wires of any desired length.

PROCEDURE

The gauge* is a self-contained Wheatstone bridge accurately balanced to null current when there is no stress applied to the pin actuating the sensitive resistance elements of the bridge. Although designed primarily for measurement of minute displacements, it may be used for direct measurement of stresses in certain ranges. A gauge with a full range of 8 oz is well suited to measuring blood volumes of the order of 100 ml. With an input to the bridge of 6 v obtained from ordinary dry cells, there is sufficient output current to actuate directly galvanometers having

* Manufactured by Statham Laboratories, Inc., 8222 Beverly Blvd., Los Angeles 36, Calif.

motor and clutching mechanism (4) or by a condenser, thyratron, galvanometer circuit (0). A recently described cardiotachometer could be used for this purpose (12).

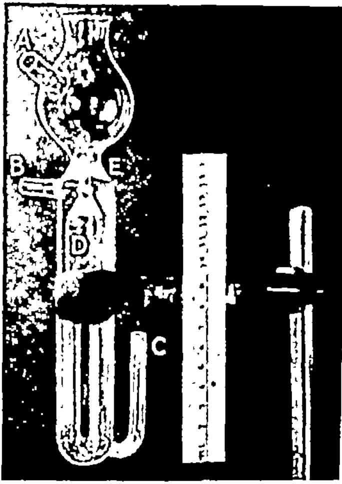

FIG. 6.—Return flow pump (Anderson (1)). Blood flowing from flow meter enters upper chamber through *A*. A slowly flowing stream of air under suitable pressure enters lower chamber through *B*. Blood trickles down through ground glass valve *E*, elevating float *D* until it seats at *E*. Air entering *B* then displaces blood out of lower chamber through connection *C* until all blood has been pumped up to the venous infusion reservoir (not shown), when float *D* drops, allowing escape of air through *E* and again allowing blood to flow from upper into lower chamber. (Reproduced by courtesy of Dr. F. F. Anderson, Ciba Pharmaceutical Products Company.)

IV. Return Flow Pumps

With all of the aforementioned meters it is necessary to return the collected blood periodically to the circulation. A convenient device for accomplishing this with minimal trauma to the blood was described by Anderson and Craver (1) (Fig. 6).

NOTE.—This section was reviewed by John R. Pappenheimer.

of the opening of the outflow tube. Although presence of slight collateral drainage of the organ under study may be regarded as a small and proportionate systematic error in some situations, the hydrostatic level of the external circuit becomes a critical determinant of the measured outflow if there are collateral routes for venous drainage (see p. 218). Excepting these reservations, however, we have found the method extremely reliable and easily adapted to a wide variety of experimental conditions.

periods of 0.1 sec. The departure from linearity of the relationship of volume (weight) to galvanometer deflection is so slight that it may be ignored for most purposes. By recording the galvanometer deflection with a conventional photokymograph, accurate graphs of the increase in volume in the outflow receiver against time are readily recorded.

In our application of this device, the outflow receiver (a beaker of 100–250 ml capacity) is placed on an aluminum platform 8 × 10 cm. The platform is suspended on three points, two of which are steel pins pivoting on tapered bearings; the third is the actuating pin of the strain gauge. If quantitatively reproducible records are desired, provision must be made for accurate placement of the receiver at a fixed point on the platform so that the mechanical advantage of suspension remains constant. It is simple to place the receiver in any convenient position on the platform and then, after each recording, to calibrate the record by withdrawing an accurately measured volume from the receiver and record the resultant galvanometer deflection before the receiver is moved. To permit selection of different recording sensitivities, a variable resistance has been placed in the galvanometer circuit with due provision for maintaining proper damping.

Adaptability of this device depends on volume flow and length of time over which a continuous record is desired. Blood collected in the receiver of the strain gauge meter is at the expense of the animal's blood volume, and if this lowers arterial blood pressure the dynamics of the circulation through the organ under study will be altered. In general, a 15 kg dog in good condition can withstand hemorrhage of as much as 100 ml of blood at the rate of 50 ml/min with only minimal fall in arterial pressure. At flows significantly below this level recordings of 3–5 min will not interfere with the arterial pressure head. To maintain pressure at higher rates of flow we have introduced into the arterial system a compensating reservoir adjusted to the proper hydrostatic level to maintain the desired pressure (see p. 120). In this way we have recorded flows of over 200 ml/min for intervals of 30 sec without change in arterial pressure.

Critique.—One particularly advantageous feature of this method in experiments for which it was designed is that the record is a cumulative measurement. Not only does the slope of the tracing at any point give the rate of blood flow at that time, but the total volume flow during an interval in which there were rapid changes in rate of flow may be measured directly on the original record without the necessity of integrating a large number of individual rate determinations.

This device does not circumvent certain problems common to all applications of the phlebotomy method. The outflow connections should be of as large a diameter and as short as practical to avoid significant resistance to flow in the external circuit, especially at high rates of flow. This precaution is easily satisfied here because of the extreme simplicity of the outflow circuit. Consideration must be given to control of collateral venous drainage, and special attention should be paid to the hydrostatic level

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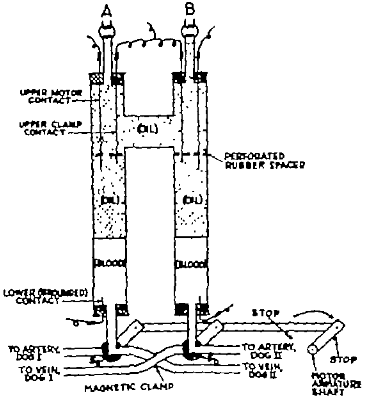

FIG. 1.—Cross-transfusion apparatus using Ludwig stromuhr principle. Blood from artery of dog II enters chamber B through stopcock S_1, displacing oil into chamber A which in turn displaces blood into vein of dog II. When rising column of blood reaches contact at top of tube B, suitable relays operate to cause the motor (lower right) to rotate to left, thus rotating the stopcocks through a 90° angle. Blood from artery of dog I now enters the bottom of reservoir A and displaces blood from reservoir B into vein of dog I. When blood in chamber A touches the upper contact the motor is again activated, rotating the stopcocks to right. (This and Fig. 2 reproduced by courtesy of *Review of Scientific Instruments* [16:95, 1945].)

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MEAN FLOW RECORDERS

HAROLD D. GREEN

Mean flow recorders suitable for insertion in the blood stream may be used in the path of venous flow where the blood returns directly to the heart or to the reservoir of a perfusion system. They have the possible advantage over outflow meters of being sensitive to variations of central venous pressure. The various mean flow meters with a linear calibration include Ludwig type stromuhrs, bubble meters (pp. 80 and 193) and similar devices which record passage of a given volume of fluid regardless of rate. Those with nonlinear calibration include thermostromuhrs (p. 80), rotameters (p. 96) and differential manometers (p. 101). Calculation of mean flow from pulsatile flow curves is possible, but is complicated by the necessity of reconstructing the curves with linear ordinate scales. An expansion chamber facilitates calculation of mean flow by damping the flow pulsations (see p. 100).

I. Ludwig Type Stromuhrs

Ludwig type stromuhrs have had many applications (4–6, 7, 8, 10). A modification has been used for cross-transfusion purposes (Figs. 1 and 2). They are satisfactory for continuous flow measurement when mean rates of flow over periods of 30–60 sec are desired. Rates of flow which fluctuate at shorter intervals are difficult to obtain with accuracy with this type of device. Unless care is used in their construction, considerable pressure drop may occur through them owing to the lengths of tubing, stopcocks and angles through which the blood flows.

II. Moving Piston Meters

Numerous moving piston meters have been devised. The author has had no experience with them. It would seem likely that they would offer considerable resistance to flow if inserted directly into the path of blood flow (1–3, 5a, 9).

cannula leads are arranged to pivot radially from the stationary meter. Kinking or tension on the artery is a cause of subnormal flows.

The meter inherently damps the central pressure pulse owing to the air in the system and gum tubing at joints. It also originates and alters reflected pulses unpredictably. For this reason coronary flow measurements (2), using the femoral artery as the central supply, have as much physiologic justification as if the central stump of the coronary, carotid or renal

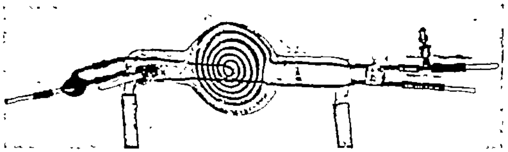

FIG. 1.—Spiral type of bubble meter showing injecting valve (upper right), marks on measuring tube, trap and straight return tube. Rubber tubes in foreground convey water at 38 C through jacket which surrounds most of the circuit.

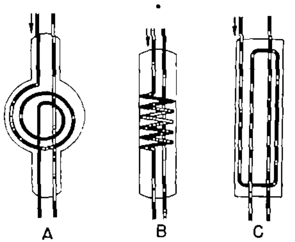

FIG. 2.—Three arrangements of measuring tube (spiral, *A*; helical, *B*; rectangular, *C*) designed to secure compactness and utility. Straight return tube is to right in each instance.

arteries were taken. As the damping cannot be entirely eliminated, a relatively constant, moderate amount seems preferable to a small but variable degree.

To obtain compactness the measuring tube, which may be 1 m long, has been coiled (Fig. 2) and ring-sealed into a jacket through which circulates water at 38 C under thermostatic control. When the meter is interpolated along an artery, the trap (Fig. 3, *A* or *B*) bridges to a straight return tube also ring-sealed into the jacket. When some other

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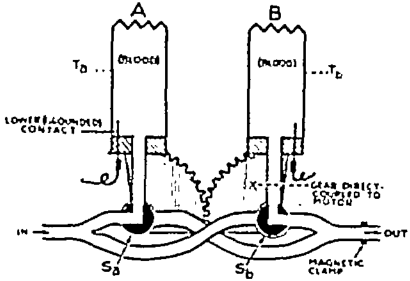

FIG. 2.—Modification of stopcocks so that apparatus (Fig. 1) may be used as a stromuhr. In the first position, blood from the vessel enters chamber A through stopcock S_a, displacing blood out into vein from chamber B through stopcock S_b. When chamber A is full, the motor is activated to rotate stopcocks 90° to allow blood to enter from left through stopcock S_b into chamber B and allow blood to leave chamber A through stopcock S_a via tube B.

III. Bubble Flow Meter

H. D. BRUNER, *University of Pennsylvania**

The original bubble flow meter (5) was a bent length of glass tubing of known volume with a trap to remove the air bubble which was injected to mark off the forward movement of blood. Subsequent refinements (1, 4) (Fig. 1) have made it a convenient direct-reading flow meter, although still imperfect in some respects. Services of a professional glass blower are desirable but not necessary. The bubble meter's advantages are self-evident, and no other stromuhr, except possibly the electric rotameter, approaches its reliability and utility.

The meter is not adapted to use in man or in chronic experiments, since the artery to be studied must be ligated and cannulated and the blood made completely incoagulable. General anesthesia is superior to local anesthesia because the meter and leads should sustain very little movement. When movement is unavoidable, as with the splenic artery, the

* At present at the University of North Carolina.

nificant pressure loss. Figure 4 shows the pressure loss at different flows due to a pair of cannulae appropriate to a *meter* of 4 ml volume.

2. Dead-end T-tubes. One T-tube is necessary to take the bubble injector; another, for injection of drugs, is optional. This appears to be a minor source of loss.

3. Tube length. In agreement with Poiseuille's law, pressure loss is proportional to length of tube, the diameter remaining constant. Theoretically this source may be minimized by selecting a larger diameter of tube, but in tubing of diameter greater than 5 mm, the bubble, even a very

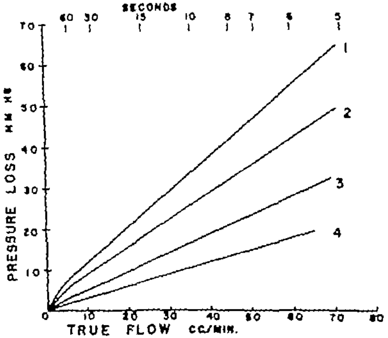

FIG. 5.—Pressure loss with respect to flow in various forms of meter: curve *1* with spiral tubing (see Fig. 2), curve *2* with helical, curve *3* with rectangular and curve *4* with straight tubing. All meters were essentially identical, having metering volumes of 5.87 ± 0.09 ml. Scale at top gives actual transit times for a bubble at flow below. Relative viscosity was 4.9 ± 0.05; temperature 24 C.

large one, spreads over a layer of blood instead of interrupting the column; the result is "slippage" and unreliable readings. Taking 5 mm tubing as the upper limit of diameter, the peak flow in practice becomes about 200 ml/min.

4. Curves in the system. Any deviation from straight-line motion occasions energy loss in moving liquids. The importance of this in design of a bubble meter is shown in Figure 5. Three of the meters had the forms shown in Figure 2—spiral, helical or rectangular; the fourth was a straight tube. The meters were identical in all other respects: total length, diameter, trap and four unavoidable 90° bends. The four meters had approximately the same minor irregularities of bore, losses from

artery is the supply, the exit cannula to the artery under study swings directly from the trap (Fig. 3, *C*) which connects directly to the distal end of the metering tube.

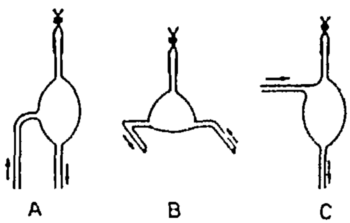

FIG. 3.—Three types of bubble traps. *A* and *B* are used where the trap bridges to the return tube. *A* is used as in Figure 1, where ends of the meter are bent 90° into a vertical plane, and *B*, where ends remain straight. *C* is used for direct connection between horizontal end of measuring tube and perfusing cannula. Bends in *B* are gentle, and not as acute as suggested here; *B* is preferred to other forms.

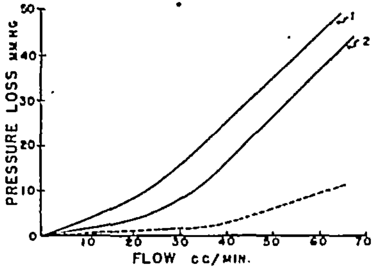

FIG. 4.—Solid lines show loss of pressure at different flows from a pair of cannulae with moderately fine throats, about 1 mm: curve *1*, with blood of relative viscosity 4.9; curve *2*, with water. Broken line shows pressure loss from a larger pair of cannulae, about 2.5 mm, using water.

From the standpoint of hydraulics (3), pressure losses arise from four conditions inherent in the meter.

1. Sudden changes in cross-section, either constrictions or enlargements. These are found in the cannulae, at joints, at irregularities of tubing from inept glassblowing and in the trap. With high flows turbulence is especially likely to develop at these points, with introduction of sig-

crease in viscosity by stabilizing streamline flow tends to reduce pressure losses from turbulence at the sites mentioned above.

A more serious effect of viscosity is related to calibration. The meter overestimates true flow (Fig. 6). The error is negligible in lower flow ranges but increases steadily in the upper ranges, and increased viscosity makes the deviation more pronounced. The effective or working caliber of the tube decreases with increasing velocity; i.e., the volume determined by static calibration fails to take into account thickening of the more or less static peripheral layers of fluid when the velocity profile elongates

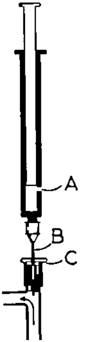

FIG. 7.—Convenient bubble injector. Hole is drilled through barrel of a tuberculin syringe, A, at a point such that injected air volume will create a gap of 1–2 cm in blood column. A 20 gauge needle, B, is closed at tip by silver solder, and lumen above opened by filing through wall. Sleeve of fine rubber tubing is fitted to make a Bunsen valve after needle has been thrust through a rubber vial stopper, C, which closes one arm of a T-tube connecting to a meter.

True flow is further overestimated when too large or too small bubbles are used: the former presumably reduce total viscous frictional resistance; the latter appear to center themselves in the tip of the velocity profile and so gain a little on mean velocity. Thus, to allow the bubble to orient itself, the starting mark is placed a fair distance downstream from the bubble injector. As standard practice, a bubble making a clean gap of 1–2 cm is secured by means of the apparatus shown in Figure 7.

Whereas deviation from true flow may be corrected by an appropriate calibration chart, there is no way of compensating for pressure loss.

which were probably negligible (*vide supra*). Obviously a compromise must be made between compactness and pressure loss from curved channels.

Meter, cannulae and connections should be designed with regard for the fact that pressure loss at bends is least when: (*a*) ratio of radius of the bend to radius of tube is between 5 and 10 or, better, greater than 30; (*b*) the angle through which the bend is carried is minimal—but total curvature (up to 90°) should be continuous rather than the sum of several discrete bends; (*c*) velocity of flow is least.

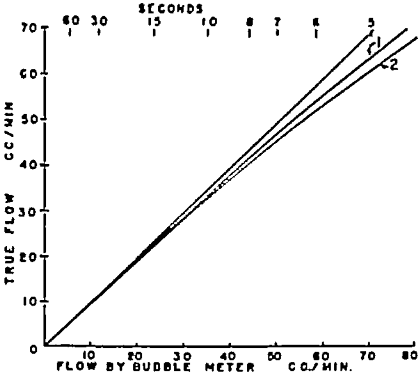

FIG. 6.—Calibration curve for meter in Figure 1. Deviation from true is shown by divergence of curves *1* and *2* from straight line sloping at 45°. Curve *1* was obtained using water and curve *2* using blood at 4.95 relative viscosity, both at 24 C. True flow was measured by collection of effluent. Scale at top is transit time of a bubble at flows below.

In conformity with Poiseuille's law, pressure loss across the meter varies directly with relative viscosity over its useful flow range. Since blood viscosity varies in different animals and may change during an experiment, it may be desirable to estimate concomitant changes of pressure loss. This is accomplished with reasonable accuracy by charting (for a given meter) pressure loss against relative viscosity and/or water at specified temperature: fractional changes of relative viscosity induce proportional changes in pressure loss. Measurement of blood specific gravity or a hematocrit reading is reasonably satisfactory for checking viscosity if viscosimeters are not available. On the other hand, an in-

quickly slipping in a cannula. The preferred cannula for this is made of thin silver tubing with a fine rolled-in groove. With practice, cannulation takes 15–30 sec, hardly enough to produce significant vascular reactions or tissue damage. On the other hand, some 3–8 min is required to intercalate a meter in a vessel, and such a connection is avoided because of probable tissue damage, regardless of presence of a collateral circulation.

Deviation from true flow and the amount of pressure loss in the meter may be obtained by in vitro calibration; with each meter assembled as it is to be used and connected to a perfusion system, one measures simultaneously the time of bubble transit and the volume discharged per unit time into a volumetric flask or accurate graduate. Blood is preferred for calibration, but solutions having a relative viscosity of 4–5 may be substituted. Since calibration values on pulsatile flows produced by a wide range of pulse rates and stroke volumes fit perfectly on a curve constructed from steady flow data, an elaborate artificial circulation is unnecessary. Pressure loss determinations may be carried out at the same time. Carefully machined pairs of brass T-tubes larger in caliber than the rest of the system are inserted proximally or distally to the cannulae depending, respectively, on whether only the meter or the total pressure loss is desired. The side tubes of these inserts connect by liquid columns to paired mercury manometers or other type of manostat. Loss of pressure at a given flow is the difference between the net pressure changes of the two manometers, taking zero flow as the point of reference. Alternatively, if pairs of inserts, liquid columns and manometers are carefully matched to eliminate extraneous hydrostatic forces, the pressure difference may be read directly; this can be used in vivo. Differential manometers are still more convenient and may be used to determine pressure loss throughout an experiment. It is worth recalling that pressure just beyond the tip of the exit cannula, and not the systemic mean blood pressure, is the true perfusing pressure.

Critique.—It is impossible to make a dogmatic statement concerning over-all accuracy of the method. The following estimates are from data of experiments where the flow might be expected to remain constant: with slow flows, total error was 1–2 per cent; with moderate flows 2–4 per cent, and with fast flows 2–8 per cent. In a controlled circulation schema the variation in a series of readings was ±2 per cent until the time of transit of a bubble became less than about 8 sec, whereupon the variation increased sometimes to ±5 per cent.

Various refinements have been considered; a few have been investigated, but discarded. The special virtue of the bubble meter lies in its ruggedness, simplicity and freedom from maintenance of adjustments.

A properly calibrated instrument will provide absolute flow data, with an estimated error of rarely more than 5 per cent. Flow through the meter entails some pressure loss, but this may be kept to a reasonable minimum by designing the meter for the flow to be studied with regard for certain principles of hydraulics.

These two tend to counteract or compensate each other in vivo, but the resultant cannot be predicted. It is preferable, therefore, to use a meter of such volume that the fastest flow shall not take less than about 10 sec between marks. This avoids the range at which errors and losses become serious. In addition, the personal error in timing transit of a bubble becomes more significant with the shorter intervals. The chronometer of choice is a 6 or 60 rpm synchronous electric timer activated by a toggle switch; a signal magnet activated simultaneously helps co-ordinate flow changes with time and other data on the record. Changes of flow occurring while a bubble is in the meter are obvious to the eye and make an indelible impression in teaching demonstrations.

PROCEDURE

To select and design a meter for a particular vascular bed, estimate the maximal flow per minute to be encountered and take one-sixth, or roughly 15 per cent, as the volume to be included between marks on the measuring tube. This volume of water is placed as a continuous column in glass tubing of suitable diameter (*vide supra*) and temporary marks inscribed. The marked-off length, with a few extra centimeters at each end, is then worked to desired form. Although the coiled shapes provide greatest compactness, pressure loss is least with some variation of the simple, elongated U-shape. A continuous circular shape, if the radius is large, occasions only slight loss, but the form is clumsy and difficult to fit into a water jacket. Necessary bends in the meter or cannulae should be smooth, perfect in cross-section and conform to the relationship already noted. Vertical bends should be avoided, since bubbles tend to climb toward or hang at high points under certain conditions. The meter should be clamped in the horizontal position. Before it is encased in its water jacket the marks are made permanent and the volume (walls wet but thoroughly drained) is carefully determined by water run in from an accurate buret.

The meter and other glass parts are kept clean by dichromate solution, traces of which are removed by sucking through quantities of $NaHCO_3$ solution. Final assembly is cleaned by drawing through distilled water and sterile saline. Glass-to-glass connections are made where possible, using the highest grade of heavy-walled pure gum rubber; it is carefully cleaned and dried following use. The importance of this cleaning and rinsing routine can hardly be overemphasized. Fibrin or platelet deposition in the tubing has not been encountered using 300 Toronto units/kg/hr of heparin (Abbott) intravenously.

It is desirable, when possible, to bridge the meter between a large supplying artery and the stump of a large branch of the artery under study. The meter then is filled from both ends and the blood supply routed through the meter by ligating the recipient *proximal to the cannulated branch*. If there are no branches, as with the renal artery, the meter is first filled from the donor vessel and clamped in correct position for

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IV. Thermostromuhr

DONALD E. GREGG, *Fort Knox, Ky.*

The thermostromuhr is a device to measure rate of mean blood flow. The earlier high frequency current models (2, 5, 8, 9) have been replaced in part by direct current types (1, 4, 10, 11). The advantage of the instrument is that it can be applied to unopened vessels of animals and readings taken as desired in the unanesthetized state.

Principle.—When a circuit is formed of two wires of different metals and one of their junctions is at a higher temperature than the other, an emf is produced in the circuit. In practical application of this principle to a flow meter, a heating element and two thermojunctions are mounted either in a rigid insulating sleeve snugly fitted to an unopened blood vessel or in a cannula inserted between the cut ends of the vessel. The thermojunctions (upstream or cold, and downstream or hot) are placed, respectively, above and below the heater, or the downstream junction is attached to the heater. The rate of blood flow is related to the differential temperature of the two junctions.

The mechanism whereby changes in differential temperature of the thermal junctions reflect changes in flow has been partially elucidated. In the type of thermostromuhr (Fig. 1) in which the cold and hot junctions are mounted with the heater in the same insulating block (1, 2, 11), both junctions are heated by the heater largely through the block and are hotter than the passing fluid; hence the differential temperature of the two junctions is determined by the respective rates at which they are cooled by the passing fluid (10, 13). In types (Figs. 2, 3) in which the cold junction is mounted separately from the hot junction and heater (4, 10), the cold junction and incoming fluid are presumably more nearly of the same temperature and the hot junction records all or a constant proportion of the temperature rise sustained by the fluid as it passes the heater. In either instance, since the cross-section of the vessel is held constant, the volume of flow is related to the differential temperature of the two junctions, provided environmental factors do not alter this relationship.

APPARATUS

Only direct current models are considered here.

The Baldes and Herrick unit (1) consists of a thin cuff of bakelite about 1 cm long which fits snugly on a blood vessel and on the inner walls of

Comment by Hiram E. Essex

This is an excellent presentation of the bubble flow meter, including the frank statement of its shortcomings as well as its advantages. The necessity for general anesthesia for cannulation of the blood vessel and use of anticoagulants seriously limits its usefulness. Its advantage over the Ludwig stromuhr and similar methods resides in its greater ease of operation. The greatest disadvantage, in our hands, was the decrease in blood flow with time in the absence of any apparent change in dynamics of the animal's circulation. The flow was characteristically rapid and smooth initially, but after 15-30 min of operation there was a decided decrease in flow rate and change from a smooth to a jerky or pulsatile flow. This was true in the presence of more than the amount of heparin recommended. Cleaning of the flow meter resulted in improved flow for 10-20 min, after which a decided decrease in rate recurred. To reduce to a minimum fibrin formation on the walls of the glass tubing, the walls were coated with silicone or the flow meter was made of polythene tubing. Even so, the difficulty persisted. Dr. Bruner does not state whether he has observed a decreased flow as the experiment is continued. This result may have been peculiar to our set-up, or his observations may have been confined to a short period subsequent to placing the flow meter in circuit. One must consider both the good and the bad features of a method, and if the advantages do not outweigh the disadvantages, it would be inadvisable to employ the method in a prospective investigation.

There is a tendency to assume that the data obtained with a given method of measuring blood flow represent, within a certain percentage of error, the real flow or the flow in the vessel when the flow meter is not present. Actually, the only certainty is that one is measuring the flow that passes through the instrument. At best, data on the flow of blood by any available method are only an approximation of the true flow.

These remarks are intended not to discourage the use of this direct method but rather to indicate that certain difficulties may be expected. Eventually a method may be developed that will have a minimum of disadvantages. The best method of measuring blood flow is yet to be devised.

Comment by Harold D. Green

In our hands, pressure drop vs. flow through cannulae has been more nearly parabolic, the lines becoming progressively more nearly parallel to the pressure axis as rate of flow increases, rather than assuming a straight line such as is shown for curves *1* and *2* in Figure 4.

Stehle (J. Pharmacol. & Exper. Therap. 46: 471, 1932) described a method in which a bubble of toluidine, colored with iodine, moves across a horizontal tube placed in front of cross-section paper. Movement of the drop is recorded with a motion picture camera. This arrangement allows measurement of both "instantaneous" and total flow.

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the hot junction lies uncovered at the downstream end of a semicylindrical plate of silver 0.20 in. long and 0.0018 in. thick. Under this plate, and insulated from it by a thin layer of mica, lies a strip of nichrome about 0.040 in. wide, 0.0018 in. thick and 0.20 in. long; to it copper leads carry the current from a 6 v storage battery through a rheostat and ammeter. The thermocouple lead wires are of silver and connected to a moving coil galvanometer with a suitable dry cell potentiometer system to permit convenient setting of the galvanometer image on the scale. When a blood vessel is placed in the unit and the silver plate is heated by passing a suit-

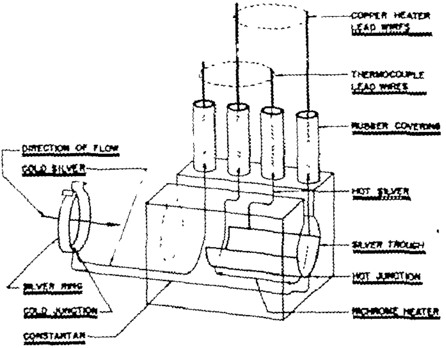


FIG. 2.—Direct current thermostromuhr. (Modified from Schmidt and Walker, *Proceedings of Society for Experimental Biology and Medicine.*)

able current (2–2.5 amp) through the nichrome strip, the extent to which the silver plate is cooled by the blood stream varies directly with the volume of blood flow.

In the thermostromuhr of Bennett, Sweet and Bassett (4), thermojunctions and heater are mounted in a cannula onto which the severed blood vessel is tied and through which passage of blood cools a heated thermojunction mounted on the end of a spicule projecting into the lumen of the device (Fig. 3). This has been used only in anesthetized animals whose blood has been rendered noncoagulable. The hot and cold junctions are made of copper-constantan or chromel-constantan (0.0015 or 0.0031 in. diameter). Under the hot junction is a nichrome wire (0.0015 in. × 0.080 in. long) soldered at its midpoint to the hot junction. The two thermocouple wires and the two portions of the nichrome heater on

which lie a heating unit and thermojunctions (Fig. 1). The heating unit is made of no. 36 or 38 nichrome wire, rolled to a ribbon 0.030-0.020 in. wide. This is folded and its two ends soldered to no. 38 copper wires, resistance of the loop being at least 1 ohm. Thermojunctions are made by soldering copper wires (0.0010 in.) to a constantan wire (0.002 in.) which is embedded in a bakelite groove, and each is placed 1.2 times the internal diameter of the cuff from the heater. The heating unit and thermojunctions are secured by several applications of bakelite lacquer* and the copper wires of both are soldered to braided copper wires (17 strands 0.003 in. tinned copper). The braided lead wires are inserted through rubber tubing and fixed with lacquer in the bakelite block.* The heater is

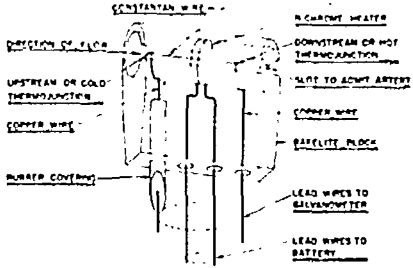


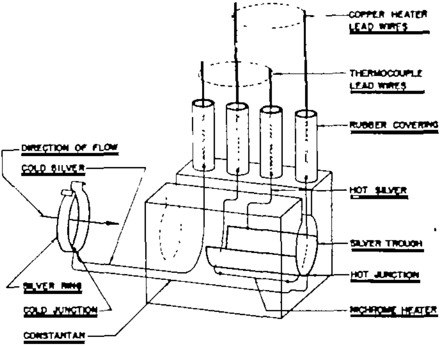
FIG. 1.—Direct current thermostromuhr of Baldes and Herrick.

connected to an electric circuit containing a 2 v storage cell, a variable resistance and a milliammeter capable of indicating currents up to 500 ma. The thermocouple is connected directly to a moving coil galvanometer, suitable characteristics being one with a low coil resistance and sensitivity of 0.5 × 10^{-6} v/mm for a scale distance of 1 m. In use the unit is placed on an unopened vessel of an anesthetized animal, the slit through which the blood vessel enters the unit is closed by a bakelite cap, the overlying tissue is replaced and the incision closed. It can be sterilized and used in chronic experiments.

In the device of Schmidt and his co-workers, the thermojunctions are of silver-constantan, and either or both are placed in a bakelite block (11) or the upstream junction is incorporated in a split silver ring attached to the bakelite block only by no. 30 silver and constantan wires 0.20 in. long covered with woven fabric insulating tubes (10) (Fig. 2). In both types,

* Substitution of bakelite lacquer XV-14463 as the insulating covering and use of 8-10 strands no. 36 or 37 annealed silver wire for lead wires reduces considerably the mechanical and electrical difficulties with the unit in chronic experiments.

the hot junction lies uncovered at the downstream end of a semicylindrical plate of silver 0.20 in. long and 0.0016 in. thick. Under this plate, and insulated from it by a thin layer of mica, lies a strip of nichrome about 0.040 in. wide, 0.0016 in. thick and 0.20 in. long; to it copper leads carry the current from a 6 v storage battery through a rheostat and ammeter. The thermocouple lead wires are of silver and connected to a moving coil galvanometer with a suitable dry cell potentiometer system to permit convenient setting of the galvanometer image on the scale. When a blood vessel is placed in the unit and the silver plate is heated by passing a suit-



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each side of the hot junction are folded back to form a pyramid with the junction at the apex; the two wires at the cold junction are similarly fashioned into a compact tip. The hot and cold tips are then mounted within and on opposite sides of a metal or lucite cannula with the cold junction 0.010–0.160 in. upstream; each tip is in a hole drilled through the wall of the cannula with the actual thermal junctions projecting equally about one third of the diameter into the lumen of the cannula. The hot junction is covered with bakelite containing powdered quartz and the two tips are fastened in place and insulated with bakelite lacquer

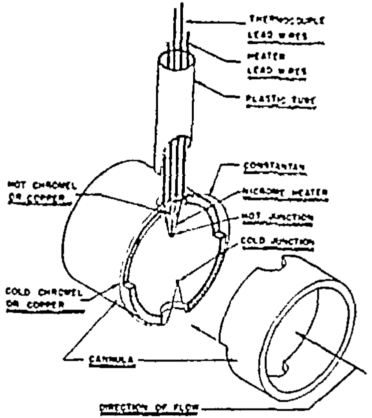

FIG. 34.—Direct current cannula type thermostromuhr of Bennett, Sweet and Bassett.

which also provides a continuous plastic lining to the cannula. The no. 28 copper lead wires pass outside the cannula. They are enclosed in stiff insulation for about 1 in. from the cannula and are then spiraled loosely around a steel stay wire (not shown) which, in turn, is looped around and soldered to the cannula. The lead wires are jacketed with plastic tubing. All wires are anchored and separated from cannula, stay wire and each other with bakelite lacquer. The thermocouple leads connect in series with a moving coil galvanometer, resistors equivalent to the critical damping resistance of the galvanometer and a suitable compensating potentiometer system to compensate for stray currents in the circuit and shifts in zero point. The relatively large basic galvanometric deflection in

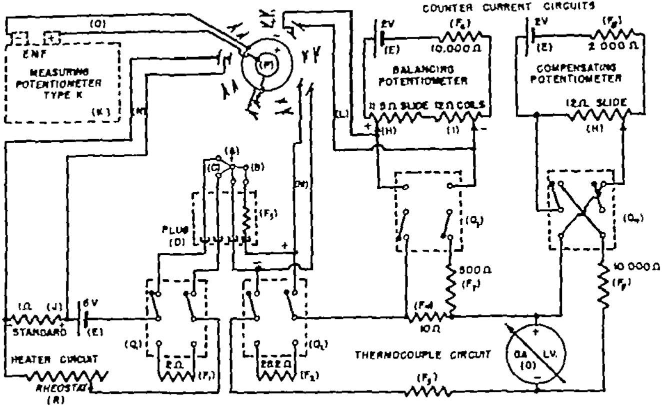

FIG. 3B.—Circuit of Bennett, Sweet and Bassett thermostromuhr. *A*, hot junction; *B*, cold junction; *C*, nichrome heater wire; *D*, 12-prong Jones plug with contacts shorted into 4 groups of 3 each; *E*, Willard low discharge storage cells (type DH-5-1 or DD-5-3); *F*, manganin resistors; *G*, Leeds & Northrop (L & N) type HS (no. 2284-A) moving coil d-c galvanometer; *H*, L & N Student type slide wire; *I*, General Radio or L & N decade resistors; *J*, L & N no. 4205 1 microhm standard resistor; *K*, L & N type K potentiometer; *L*, leads for measuring countercurrent emf; *M*, leads for measuring thermocouple emf; *N*, leads for measuring heater current; *O*, common measuring lead; *P*, L & N no. 8240 double pole 10-throw rotary selector switch; *Q*, L & N no. 3284 DPDT knife switch; *R*, decade and slide wire rheostat. (Redrawn from Bennett *et al.* (4).)

the thermocouple circuit resulting from heating of the hot junction is balanced out by a potentiometer in series with a 2 v storage cell. The heater wires are connected in series with rheostat, ammeter and storage battery.

CALIBRATION

Blood flow values are determined by applying a calibration curve to the deflections of the galvanometer as recorded during an experiment. An in vitro calibration is obtained by perfusing blood (rendered non-coagulable) in a gravity or pump system through the cannula type of thermostromuhr or through an isolated segment of blood vessel to which a thermostromuhr is snugly fitted. Flow is measured in a graduate or by a rotameter (7, 12) in the circuit. At the close of an experiment, calibration can be made in vivo by means of a rotameter or other acceptable metering device inserted into the vessel distal to the thermostromuhr and with flow adjusted to various rates by a screw clamp (see p. 96).

Critique.—Tests in vitro and in vivo have revealed serious deficiencies in application of this thermal principle to measurement of blood flow (3, 4, 6, 10, 13, 14). Several external and internal environmental factors other than rate of blood flow influence significantly and unpredictably the empiric flow readings by altering relative rates of heat loss to the environment at the two junctions or heat distribution between the junctions. These basic limiting factors include the artery used, angulation of the unit with respect to the artery, movements of intra- and extravascular fluid near the unit, lack of linearity between temperature changes within unit and blood flow, changes in blood temperature, uneven temperature in various parts of the blood stream, occurrence of backflow within the flow pattern. The amount of backflow in the flow pattern is particularly important since the flow pattern in most arteries either normally contains backflow or it can be induced, and the instrument is so sensitive that such a change can cause the device to indicate incorrectly the directional change in flow. Although in some models errors have been reduced by various expedients, such as separation of the cold junction from the rest of the unit (10) or placement of thermocouples directly in the flowing stream (4), most of the basic errors remain. Because of this, the conclusion is reached that such a thermal method in its present state of development is unsatisfactory for measuring blood flow.

Comment by Carl F. Schmidt

The thermostromuhr has a seductive appeal to physiologists because it is reputed to possess the following unique attributes: (*a*) accurate, quantitative measurement of volume of blood flowing through an artery or vein; (*b*) avoidance of anesthetics, anticoagulants, extravascular diversion of blood or other departures from the normal state; (*c*) continuous estimation of blood flow under either steady state or rapidly changing conditions; (*d*) simultaneous measurement of flow in several different vessels. Any one of these would entitle the method to

careful consideration; the combination is unequaled by any existing method or, indeed, any combination of other methods.

It is disappointing to have to admit that the thermostromuhr is unreliable quantitatively and is not even entirely dependable qualitatively. This conclusion has been forced on all who have made a systematic attempt at in vivo calibration of the instrument (4, 6, 10, 13). The fundamental difficulty is that difference in temperature between the thermal junctions depends on the algebraic sum of a number of different variables, of which blood flow through the contiguous vessel is only one, and not always the dominant one. Some of these variables have been identified and evaluated by Gregg and his co-workers (6). There probably are others. For example, it would be remarkable if conduction of heat through an artery in vivo, with its wall supplied with blood carried through vasa vasorum, would be even approximately similar to that through an excised artery in which flow through vasa vasorum is precluded by cannulae tied at its two ends for a perfusion calibration in vitro. Conduction of heat from the bakelite block of the thermostromuhr to the tissues with which it is in contact also may be entirely different in vivo and in vitro. The conclusion is inescapable that the only acceptable calibration of a thermostromuhr is one made under conditions that do not differ in any important respect from those under which the experimental observations were carried out. Such calibrations are practicable only in a few regions, one of the best being the kidney. Any experimenter who plans to use the thermostromuhr would do well to carry out a set of in vivo calibrations on the renal circulation before he commits himself to the vagaries of the instrument.

Comment by Harold D. Green

An additional reference is Tvede-Jacobsen, J. K.: Method of determining the quantity of heat evolved on the complex resistance in an element of the bloodflowmeter ("thermostromuhr") after Rein, Acta physiol. Scandinav. 2: 249, 1941.

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V. Rotameter

R. E. SHIPLEY, *Indianapolis General Hospital*

The rotameter (5) is useful in physiologic studies requiring measurement of mean blood flow in either arteries or veins (2–4, 6, 7, 9, 10). The method is simple and accurate and permits continuous observation of changes in flow from moment to moment.

APPARATUS

In its simplest form the rotameter consists of a vertical transparent tube with tapered bore (Fig. 1, *A*). In the tube is a small, freely movable float which rests on a stop at the bottom when no fluid is passing through the rotameter. With upward flow through the tube the float is lifted by the stream until it reaches a height determined by a balance of the downward force (weight of float minus weight of displaced fluid) and the upward force (pressure drop across float times maximal cross-sectional area of float). If the flow is then increased the balance is disrupted and the float rises to a new level, at which position the annular orifice is just large enough to reduce the pressure drop across the float to that existing at the previous flow level.

For notation of position of the float, millimeter graduations are marked on the rotameter tube. The instrument is calibrated by plotting height of the float against outflow as measured with a graduate and stop watch.

Theoretically, vertical displacement of the float would vary linearly with the rate of flow if it were not for viscosity effects. However, at the lower flow range the float is lifted a disproportionately greater amount than at the higher range, and the calibration curve is consequently nonlinear (Fig. 1, *B*). Variations in viscosity of fluid being metered will induce corresponding variations in calibration. In physiologic use the rotameter should be calibrated for each experiment and at least several points checked during an experiment if the conditions imposed are suspected of causing hemoconcentration or hemodilution.

Essential steps for construction of the simplest form of rotameter fol-

low.* A cylindrical piece of lucite or other transparent plastic is drilled through the center, with care to avoid overheating of the drill and consequent cracking of the lucite. The hole is reamed through its entire length by hand, using a standard taper pin reamer, the end of which is slightly larger than the drilled hole. Removable inlet and outlet fittings (see Fig. 1) are made from a nonrusting metal. The float, turned on a lathe to the shape approximating that in Figure 1, may be made of any nonrusting metal, or of plastic if extreme sensitivity is desired. The greatest diameter (top) of the float is 0.001–0.002 in. less than the internal diameter of the lucite tube opposite the position the float will occupy when it rests on the bottom fitting (zero flow position).

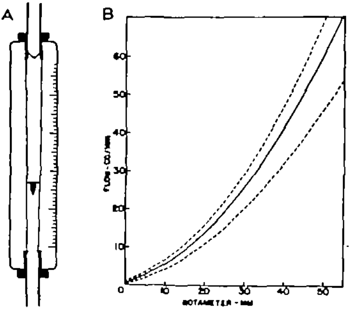

FIG. 1.—*A*, sectional view of rotameter. *B*, sample calibration curves. Solid line curve obtained with blood of average viscosity; broken lines, calibrations with bloods having less and more viscosity than average.

Design and construction of the rotameter will vary according to sensitivity desired and range of flow to be covered. By making the float of a heavy metal or by reducing its diameter, the same rotameter will have less sensitivity but a greater flow capacity. (The latter modification, however, will not permit measurement of flow in the low range.) Greater sensitivity with small flow capacity may be obtained by making the float of a light material such as aluminum, plastic, hard rubber, etc.

Using the same principles, large rotameters capable of measuring cardiac output may be constructed (5). For measuring blood flow in the

* Commercial models of the rotameter can be obtained from Fischer and Porter Co., Philadelphia.

venae cavae or other veins light floats and large bore rotameter tubes are desirable so that loss of pressure head and reduction in blood flow will be minimal.

Critique.—For physiologic experiments the rotameter has several advantages over other instruments. It is simple to construct, requires no adjustments and is easy to operate. It permits a direct, continuous objective measurement of flow with an accuracy of ±10 per cent. Calibration is not affected by changes in flow pattern (phasic directional and velocity changes in flow pulse). In common with other direct methods for measuring flow, the rotameter has the disadvantage of requiring that the blood vessel be severed and cannulated. In addition, the animal's blood must be rendered noncoagulable. If too little anticoagulant is used, a fine deposition of fibrin occasionally forms on the rim of the float and the instrument may not correctly indicate actual flow. Presence of fibrin can be detected and such errors avoided if periodically the flow through the rotameter is temporarily interrupted and the float allowed to come to rest on the stop. If the float does not fall completely to the normal zero flow position, the rotameter should be by-passed, the float removed and cleaned.

Modifications in design have been made to permit continuous optical recording of the flow (1, 8). Changes in vertical position of the float are detected by an induction mechanism, and by electronic amplification the variations in flow are ultimately recorded by means of a light beam reflected from the mirror of a galvanometer. The electrical unit has been simplified by omitting the amplifying system and employing more sensitive recording instruments (11).

Comment by Raymond P. Ahlquist

The rotameter measures *mean* volume flow only. It should be apparent from the statement, "calibration is not affected by changes in flow pattern (phasic directional and velocity changes in flow pulse)," that the rotameter in present forms cannot be used to measure these phasic changes. The slower the change in flow the more closely the rotameter follows true flow; conversely, the more rapid the change in flow the less faithful the rotameter record. This effect is due to inertia of the float and of the recording galvanometer in the optical recording rotameter. This is readily seen in arterial flow records when the heart rate is changed. With a slow heart rate, excursions of the float are wide, and with a rapid heart rate, much smaller. With a heart rate over 100 beats per minute the optical rotameter produces a line almost unmarked by phasic flow changes.

The rotameter can be used for measuring flows in other types of physiologic experiments. It is much more satisfactory than drop or "bucket" recorders for any flows which are not too small. It is conveniently used in perfusions of isolated mammalian heart and in heart-lung preparations.

In this laboratory it has been found convenient in some experiments to place a special bubble meter in series with the rotameter to allow frequent standardizations of the rotameter. This bubble meter is an adaptation of the one described by Soskin *et al.* It is equipped with a manually operated reversing valve so that only one air bubble is needed.

Comment by Harold D. Green

I have been concerned about use of the rotameter in measuring flows which pulsate over a range from zero to some figure, in the positive direction, feeling that the mean given by the rotameter would probably differ from the true mean by a significant figure owing to the nonlinearity of the calibration.

Comment by Dr. Shipley

The comments of Dr. Ahlquist are quite right. The rotameter is obviously unsuitable for measuring phasic changes in rate of flow.

The comments concerning the effects of flow pattern changes on the calibration, I believe, are answered by the experiments reported in our first paper on the rotameter (5). The instrument was surprisingly insensitive to wide fluctuations in flow pattern and the calibration (using a pump system) remained almost identical to that obtained with nonpulsating flow.

Further comment by Dr. Ahlquist

I have recognized the error of the rotameter at low flow rates and so have kept out of that range if possible. I do not consider this error, however, to be due to nonlinearity of calibration. In my experience, calibration is linear at low flow rates. With saline solution, at least, it is an absolutely straight line in this range. I consider the error to be of structural origin. E.g., if a large dose of epinephrine is injected into the femoral artery during measurement of flow therein, the rotameter float pulsates from zero to some positive figure. The backflow during diastole is not measured because construction of the rotameter prevents this. Therefore I believe the mean flow indicated is not the true mean flow chiefly because of inability of the rotameter to measure backflow. In pharmacologic experiments I therefore adjust the dosage of vasoconstrictors so that the flow is not diminished enough to cause the float to hit the bottom of the meter.

The bubble meter also shares this error at low flow rates, but for a different reason. In the same type of experiment as that just described, each pulsation forces a little blood past the air bubble. This makes it appear in some cases that the mean flow is running backward.

Although I have used only a rotameter or bubble meter, I believe only a Ludwig stromuhr or an orifice meter can give accurate flow results under these conditions. Hot wire flow meters and thermostromuhrs are undoubtedly inaccurate for flows pulsating from zero (or a backflow) to a positive flow.

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Modifications in design have been made to permit continuous optical recording of the flow (1, 8). Changes in vertical position of the float are detected by an induction mechanism, and by electronic amplification the variations in flow are ultimately recorded by means of a light beam reflected from the mirror of a galvanometer. The electrical unit has been simplified by omitting the amplifying system and employing more sensitive recording instruments (11).

Comment by Raymond P. Ahlquist

The rotameter measures *mean* volume flow only. It should be apparent from the statement, "calibration is not affected by changes in flow pattern (phasic directional and velocity changes in flow pulse)," that the rotameter in present forms cannot be used to measure these phasic changes. The slower the change in flow the more closely the rotameter follows true flow; conversely, the more rapid the change in flow the less faithful the rotameter record. This effect is due to inertia of the float and of the recording galvanometer in the optical recording rotameter. This is readily seen in arterial flow records when the heart rate is changed. With a slow heart rate, excursions of the float are wide, and with a rapid heart rate, much smaller. With a heart rate over 100 beats per minute the optical rotameter produces a line almost unmarked by phasic flow changes.

The rotameter can be used for measuring flows in other types of physiologic experiments. It is much more satisfactory than drop or "bucket" recorders for any flows which are not too small. It is conveniently used in perfusions of isolated mammalian heart and in heart-lung preparations.

In this laboratory it has been found convenient in some experiments to place a special bubble meter in series with the rotameter to allow frequent standardizations of the rotameter. This bubble meter is an adaptation of the one described by Soskin *et al.* It is equipped with a manually operated reversing valve so that only one air bubble is needed.

Comment by Harold D. Green

I have been concerned about use of the rotameter in measuring flows which pulsate over a range from zero to some figure, in the positive direction, feeling that the mean given by the rotameter would probably differ from the true mean by a significant figure owing to the nonlinearity of the calibration.

Comment by Dr. Shipley

The comments of Dr. Ahlquist are quite right. The rotameter is obviously *unsuitable for measuring phasic changes in rate of flow.*

The comments concerning the effects of flow pattern changes on the calibration, I believe, are answered by the experiments reported in our first paper on the rotameter (5). The instrument was surprisingly insensitive to wide fluctuations in flow pattern and the calibration (using a pump system) remained almost identical to that obtained with nonpulsating flow.

Further comment by Dr. Ahlquist

I have recognized the error of the rotameter at low flow rates and so have kept out of that range if possible. I do not consider this error, however, to be due to nonlinearity of calibration. In my experience, calibration is linear at low flow rates. With saline solution, at least, it is an absolutely straight line in this range. I consider the error to be of structural origin. E.g., if a large dose of epinephrine is injected into the femoral artery during measurement of flow therein, the rotameter float pulsates from zero to some positive figure. The backflow during diastole is not measured because construction of the rotameter prevents this. Therefore I believe the mean flow indicated is not the true mean flow chiefly because of inability of the rotameter to measure backflow. In pharmacologic experiments I therefore adjust the dosage of vasoconstrictors so that the flow is not diminished enough to cause the float to hit the bottom of the meter.

The bubble meter also shares this error at low flow rates, but for a different reason. In the same type of experiment as that just described, each pulsation forces a little blood past the air bubble. This makes it appear in some cases that the mean flow is running backward.

Although I have used only a rotameter or bubble meter, I believe only a Ludwig stromuhr or an orifice meter can give accurate flow results under these conditions. Hot wire flow meters and thermostromuhrs are undoubtedly inaccurate for flows pulsating from zero (or a backflow) to a positive flow.

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PULSATILE FLOW METERS

HAROLD D. GREEN

METERS CAPABLE of registering the instantaneous rate of flow are necessary for determining the flow pattern in the aorta, arteries and central veins, for analyzing factors affecting coronary blood and for studying effects of muscular contractions, sudden accelerations and other fleeting factors the effects of which occur within intervals shorter than those which can be accurately studied with mean flow meters. Suitable methods are the differential manometer, magnetic flow meter (p. 108) and air expansion systems (p. 107).

I. Differential Pressure Flow Meters

Devices for water flow measurement are directly applicable to blood flow. In general, they all involve the creation between two points in the circuit of a pressure difference which bears a constant relationship to the volumetric rate of flow per minute. A wide variety of such meters have been described (2–6, 13, 14, 16, 20).

The difference in pressure may be created by a simple constriction, in which case *the difference is due to frictional loss of energy as the fluid* flows through the constriction (Fig. 1, *A*). The orifice, venturimeter and Petôt tube principles involve devices which make use of the velocity with which the fluid is flowing and, in general, cause less over-all loss of pressure head than is the case with simple frictional devices, since pressure differences due to friction represent permanent loss of pressure energy, whereas 50 per cent or more of the pressure difference due to change of velocity may be regained.

The *venturimeter* (Fig. 1, *B*) determines the difference in lateral pressure between an upstream portion of the tube and a narrow portion downstream. The high velocity of flow through the downstream portion causes diminution in lateral pressure at this point in comparison with that at the upstream pressure point, which is approximately proportional to the square of the volumetric rate of flow. Gradual widening of the tube distal to the point of narrowing causes restoration of most of the lateral pressure which was available above the point of narrowing (3, 20).

The *Petôt tube* method (Fig. 1, *C*) involves insertion of one tube in the path of fluid flow, directed upstream, and another either flush with the lateral wall or directed downstream. This method depends entirely on velocity of flow of fluid at the point of insertion (4, 14).

The *orifice meter* (Fig. 1, *D*) depends on measurement of the difference in lateral pressure upstream and downstream from a point of narrowing. Narrowing of the stream is produced by causing the fluid to flow through an opening—the orifice—in a thin plate placed in the path of the stream flow, the orifice being slightly narrower than the general tube diameter. For detection of differential pressure, lateral piezometer openings are placed $^1/_2$ tube diameter upstream and downstream, respectively, from the orifice plate. The high velocity stream flowing through the orifice at-

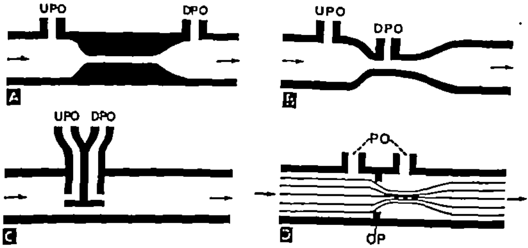

FIG. 1.—Devices used to generate a differential pressure which will vary with rate of flow. *A*, friction device in which difference in upstream (*UPO*) and downstream (*DPO*) lateral pressures depends on frictional loss of energy in region of constriction of the tube. *B*, venturimeter in which difference in lateral pressures is due to difference in velocity of flow past the upstream and downstream piezometer connections. *C*, Petôt tube device. *D*, orifice device. Narrow lines represent path of flow of fluid through and beyond orifice *OP*. Because fluid continues to converge beyond the orifice, the effective orifice diameter is smaller than the actual. *PO*, upstream and downstream piezometer connections, which are most effectively placed one-half the tube diameter upstream and downstream from the orifice plate.

tains a diameter slightly less than that of the orifice at the point $^1/_2$ tube diameter downstream from the orifice, then gradually widens back to tube diameter (Fig. 1, *D*). As a result of narrowing and acceleration the fluid at this point has a lateral pressure less than the upstream pressure by an amount approximately proportional to the square of volumetric rate of fluid flow (11).

Orifice and Petôt tube methods may be utilized in pulsating streams, even when pulsation is such that the fluid reverses its direction of flow during part of the cycle. The venturimeter may be utilized for pulsating flow, but only provided pulsations occur in one direction without reversal of flow at any time during the cycle. In our hands, the Petôt tube is less satisfactory than the orifice meter because of difficulty of construction, occurrence of vibrations apparently due to eddy currents and difficulty of

obtaining suitable sensitivity and natural frequency. For these reasons we concentrated on the orifice meter (11).

a) Orifice meter.—This has the advantage over the other devices mentioned in that any degree of sensitivity can be conveniently obtained by varying the size of the orifice. Two methods accomplish this. One devised by Shipley *et al.* (10) utilizes a screw device which protrudes into the lumen, decreasing the size of the tube between the piezometer openings.

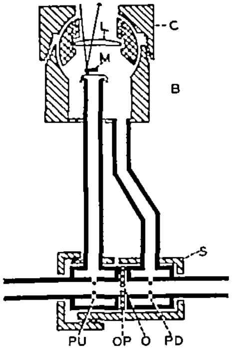

FIG. 2.—Construction details for orifice plate meter and associated differential manometer. *OP*, orifice plate; *O*, orifice; *PU*, upstream, and *PD*, downstream piezometer connections. *S*, shell to hold together component parts of the orifice; slit in shell to left of orifice plate is so constructed that by loosening cap on shell the slit can be slid over orifice plate and plate lifted out without disturbing positions of tubes connected with the piezometer opening. *B*, base of differential manometer; *L*, lens carried in a ball; *C*, cap which holds ball in place. Lens serves as window of manometer. *M*, mirror attached to rubber diaphragm of manometer.

The other involves a convenient slot in the orifice meter assembly, whereby the orifice plate may be quickly removed and another with a different size of orifice inserted (Fig. 2). The diameter of the meter tube is usually made to approximate that of the lumen of the blood vessel in which flow is to be measured.

Differential pressure of an orifice meter is equal to a constant times rate of flow plus another constant times the square of the rate of flow. The first power effect is due to friction and is influenced by viscosity. It is most evident at low rates of flow. The second power effect is due to velocity and is little influenced by viscosity. The result of this dual effect

is seen in the calibration plots (Fig. 3), demonstrating that at low rates of flow viscous effects are marked, blood gives a much greater deflection than saline and the slope tends to be 1:1. At high rates of flow, however, the plot approaches a slope of 1:2 and blood and saline cause almost identical differences of pressure.

One difficulty encountered with the small bore orifice plates is that a small coagulum, presumably of platelets, occurs in the orifice, narrowing the opening and progressively increasing sensitivity of the apparatus dur-

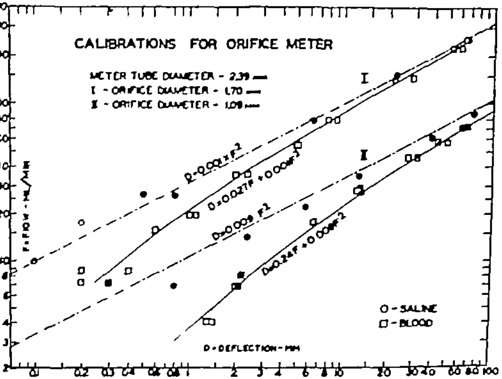

Fig. 3.—Calibrations for an orifice meter, using saline and blood and two different orifices. Dot-dash line, ideal line representing relationship between flow (ordinate scale) and differential pressures, i.e., deflection of recording beam (abscissal scale). Solid line, curve drawn in attempt to match observed points for blood.

ing an experiment despite use of large amounts of anticoagulants. To overcome this, the apparatus should be constructed so that the orifice plate can be frequently cleaned, and spot calibrations at one or two rates of flow should be taken not less than every $^1/_2$ hr.

Figure 4 shows a typical assembly for using the orifice meter (see also Fig. 2, p. 121). The arrangements consist of a shunt whereby flow may be maintained when the orifice is being calibrated, and suitable connections for running blood through the orifice at varying rates and for collecting the outflow in a graduate or other measuring device while recording the differential pressure so created (1). Since a given movement of the differential recorder means a greater increment of flow at low than at high rates

of flow, it is mandatory that frequent zero flow calibrations be made throughout each experiment. Such calibrations require only 1–2 sec interruption of flow and should be made as often as every 5–10 min.

b) Differential pressure manometers.—The aforementioned devices require a manometer which is insensitive to the absolute head of pressure but is highly sensitive to small differences in pressure between the two piezometer connections and has a natural frequency of 25–50 cycles/sec or better.

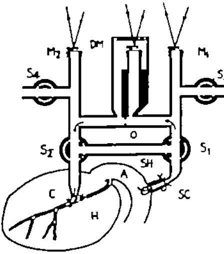

FIG. 4.—Assembly for using the orifice meter for measuring coronary blood flow. *DM*, differential manometer; M_1, and M_2, pressure manometers (M_1 can usually be omitted); S_3, stopcock for admitting blood from a reservoir; S_4, stopcock for allowing blood to flow out with a graduate for calibrating meter; S_1 and S_2, stopcocks for allowing blood circulation to heart through shunt *SH* while calibrating orifice; *SC*, cannula inserted in subclavian artery; *C*, cannula inserted in distal stump of coronary artery. (Reproduced by courtesy of *American Heart Journal.*)

APPARATUS

Such a manometer (11) is simply a rubber membrane* optical pressure manometer with a chamber surrounding the front surface of the membrane for application of counterpressure. Upstream lateral pressure from the orifice meter is directed against the back side and downstream pressure against the front side of the membrane. The membranes are constructed from 0.006 in. thick rubber membrane sheeting, stretched three to four times and tied over a hollow manometer tip approximately 4 mm in external diameter. A mirror† is cemented at the appropriate point on

* Rubber membrane 0.004–0.009 in. is satisfactory. That which we use was obtained from American Anode Company, Inc., Akron, O. When assembled in the meter the membranes usually last several days to several weeks, depending on room temperature.

† Approximately 6 × 6 mm pieces cut from U. S. Bureau of Standards certified counting chamber cover slips. Originally we used rear-surfaced silvered mirrors, the silver being protected by a covering of the special adhesive.‡ At the suggestion of Dr. R. E. Shipley, we now use front-surfaced chromium-plated mirrors prepared for us by Evaporated Metal Films Corp., 436 W. State St., Ithaca, N. Y.

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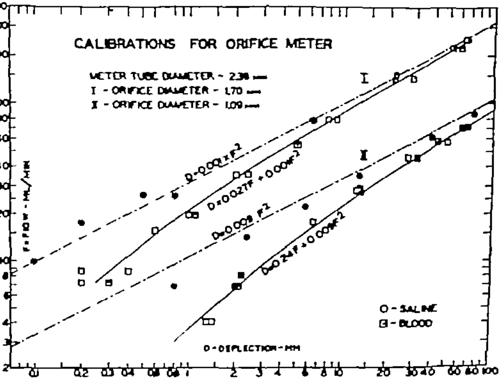

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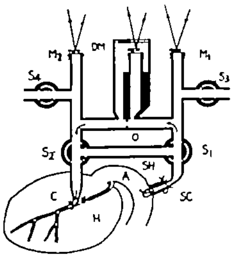

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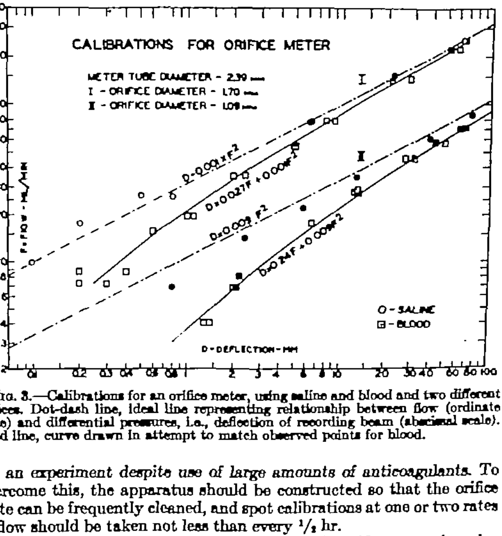

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cally recording bellows manometers (15, 16). Their principal disadvantages for venous outflow are necessity for frequent calibration and the slightly greater intrinsic resistance to flow as compared with the meters described on pages 68 ff.

II. Air Expansion Systems

Perfusion of organs with blood from a reservoir, pressure being applied by an air chamber above the blood, allows measurement of rate of flow in terms of rate of drop of pressure in the air chamber (9) or in the fluid (18, 21) measured by a suitably sensitive manometer. This method has been used for coronary flow studies and would be applicable to other situations in which relatively rapid fluctuations of flow occur.

The air pressure type of device suffers the slight disadvantage, when rapid rates of flow are being measured, that, owing to sudden expansion of air in the chamber above the blood, cooling occurs which reduces pressure more rapidly when blood is leaving the chamber. Pressure then rises slightly after flow has ceased. This difficulty can be almost completely overcome if the surface for absorption of heat from the environment is large relative to the volume of air above the perfusion fluid. When pressure in the perfusion fluid is recorded instead of the air pressure, oscillations due to water hammer effects occur which do not represent fluctuations in flow. These devices may be used to measure flow rate over intervals as short as 0.2–1.0 sec. However, the chamber requires frequent refilling with blood in order to obtain a continuous record of rate of flow over any prolonged period, and it is difficult to prevent sedimentation of the blood.

Comment by Donald E. Gregg

I have little criticism of the methods indicated or of their description. The adjustable screw for changing the sensitivity of the orifice meter devised by Shipley *et al.* is, as far as I know, an adequate instrument in operation and is much more flexible than the insertion of disks.

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the membrane by an intervening peg‡ cemented to the mirror and membrane by a self-vulcanizing rubber cement.§ The point of contact of peg with membrane should be as small as possible and should be at the point of maximal deflection of the membrane, which is usually at the point where the membrane bends over the edge of the manometer tube (Fig. 2).

A wedge of water usually present between the window of the differential manometer and the mirror causes color separation in the recording beam and therefore prevents a sharp-edged recording. This may be corrected by a properly oriented prism of suitable power, placed in front of the window (11). It is more convenient and causes less loss of light to mount the window in a ball and socket (10) (Fig. 2). Optical systems for use with these differential manometers have been described in detail elsewhere (8).

Electrically operated capacitance manometers of the type described by Lilly and his associates (17) could probably also be used for this purpose, as could the differential type of strain gauge.‖ For measurements of flow in nonpulsating streams, a direct writing differential manometer may be used (16). It may be composed of two sensitive matched metal bellows attached to a single writing point, the bellows being connected to an upstream and downstream connection of the orifice meter or other differential pressure generating device (15).

c) Use of differential pressure meters for mean flow registration.—Calculation of mean flow from pulsatile flow curves requires determination of the mean deflection. The latter is accomplished readily by measuring the area under the curve with a planimeter and dividing by the length of the base. When deflection of the recorder is not linear with respect to flow, mean deflection will give the mean flow with not more than 1.7 per cent error when minimal deflection is equal to at least one-half the maximum (12). This rarely occurs, however, and when flow approaches zero or when backflow is obtained the curve must be redrawn with a linear ordinate scale before it is integrated, otherwise large errors may be incurred. Where the meter's calibration is approximately linear with a slope of 1:2 on log-log paper, the square root extractor is very useful (7). Otherwise points along the curve must be replotted to a linear ordinate scale with aid of a calibration graph (Fig. 3).

When pulsatile flow does not occur, as in peripheral veins, or when mean flow may be produced, as in artificial perfusion systems or by insertion of an expansion chamber upstream from the meter in recording arterial inflow, as shown in Figure 2 (p. 121), more sensitive but lower frequency differential pressure manometers may be used. These may be of the optical type already described, water manometers (20) or mechani-

‡ The pegs are conveniently made from glass rod pulled to approximately 0.040 in. diameter and broken off in lengths of approximately 0.040 in.

§ The cement is special adhesive 10381 obtained from American Anode Company, Inc., Akron, O.

‖ *Statham Laboratories* Inc., 8222 Beverly Blvd., Los Angeles 36, Calif.; model P-21 pressure transmitter.

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When pulsatile flow does not occur, as in peripheral veins, or when mean flow may be produced, as in artificial perfusion systems or by insertion of an expansion chamber upstream from the meter in recording arterial inflow, as shown in Figure 2 (p. 121), more sensitive but lower frequency differential pressure manometers may be used. These may be of the optical type already described, water manometers (20) or mechani-

‡ The pegs are conveniently made from glass rod pulled to approximately 0.040 in. diameter and broken off in lengths of approximately 0.040 in.

§ The cement is special adhesive 10381 obtained from American Anode Company, Inc., Akron, O.

‖ Statham Laboratories Inc., 8222 Beverly Blvd., Los Angeles 36, Calif.; model P-21 pressure transmitter.

cally recording bellows manometers (15, 16). Their principal disadvantages for venous outflow are necessity for frequent calibration and the slightly greater intrinsic resistance to flow as compared with the meters described on pages 98 ff.

II. Air Expansion Systems

Perfusion of organs with blood from a reservoir, pressure being applied by an air chamber above the blood, allows measurement of rate of flow in terms of rate of drop of pressure in the air chamber (9) or in the fluid (18, 21) measured by a suitably sensitive manometer. This method has been used for coronary flow studies and would be applicable to other situations in which relatively rapid fluctuations of flow occur.

The air pressure type of device suffers the slight disadvantage, when rapid rates of flow are being measured, that, owing to sudden expansion of air in the chamber above the blood, cooling occurs which reduces pressure more rapidly when blood is leaving the chamber. Pressure then rises slightly after flow has ceased. This difficulty can be almost completely overcome if the surface for absorption of heat from the environment is large relative to the volume of air above the perfusion fluid. When pressure in the perfusion fluid is recorded instead of the air pressure, oscillations due to water hammer effects occur which do not represent fluctuations in flow. These devices may be used to measure flow rate over intervals as short as 0.2–1.0 sec. However, the chamber requires frequent refilling with blood in order to obtain a continuous record of rate of flow over any prolonged period, and it is difficult to prevent sedimentation of the blood.

Comment by Donald E. Gregg

I have little criticism of the methods indicated or of their description. The adjustable screw for changing the sensitivity of the orifice meter devised by Shipley *et al.* is, as far as I know, an adequate instrument in operation and is much more flexible than the insertion of disks.

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the membrane by an intervening peg‡ cemented to the mirror and membrane by a self-vulcanizing rubber cement.§ The point of contact of peg with membrane should be as small as possible and should be at the point of maximal deflection of the membrane, which is usually at the point where the membrane bends over the edge of the manometer tube (Fig. 2).

A wedge of water usually present between the window of the differential manometer and the mirror causes color separation in the recording beam and therefore prevents a sharp-edged recording. This may be corrected by a properly oriented prism of suitable power, placed in front of the window (11). It is more convenient and causes less loss of light to mount the window in a ball and socket (10) (Fig. 2). Optical systems for use with these differential manometers have been described in detail elsewhere (8).

Electrically operated capacitance manometers of the type described by Lilly and his associates (17) could probably also be used for this purpose, as could the differential type of strain gauge.‖ For measurements of flow in nonpulsating streams, a direct writing differential manometer may be used (16). It may be composed of two sensitive matched metal bellows attached to a single writing point, the bellows being connected to an upstream and downstream connection of the orifice meter or other differential pressure generating device (15).

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‖ Statham Laboratories Inc., 8222 Beverly Blvd., Los Angeles 36, Calif.; model P-21 pressure transmitter.

$$E = H \cdot l \cdot v \cdot 10^{-8},$$

where E is the potential difference in volts; H, field strength in gauss; l, length of the conductor within the field in cm (at right angles to direction of motion and lines of force in the field); v, speed of the conductor in cm/sec. If H and l are kept constant, the voltage developed will be a linear function of the speed of the conductor:

$$E = K \cdot v \qquad K = H \cdot l \cdot 10^{-8}$$

E is independent of the material of the conductor, which therefore may consist of a solution of electrolytes, such as blood. Use is made of this fact in practical application of this principle to the flow meter.

APPARATUS AND PROCEDURE

The complete apparatus consists of a magnet, insulating sleeve, two nonpolarizable electrodes, a direct-coupled amplifier and a recording galvanometer.

FIG. 1.—Two types of magnets used with flow meter.

a) Magnet.—The constant uniform magnetic field necessary for quantitative accuracy may be obtained by using a permanent magnet or an electromagnet energized by a suitable d-c source such as a storage battery or well filtered d-c line. It is desirable to produce the strongest magnetic field possible consistent with the limitations imposed on the magnet by the space available. Size and shape of the magnet and shape of the pole pieces are determined by size and location of the blood vessel to which it is to be applied. Width of the air gap is also determined by size of the blood vessel and should, of course, be as small as possible to provide maximal field strength for a given magnetomotive force. Two types of magnets which have been used are shown in Figure 1. *B* is a small electromagnet, with core constructed from laminations removed from a small filament transformer. Each coil consists of approximately 60 ft of no. 22 B. & S. enameled wire. Total resistance of the two coils in series is approximately 2 ohms. When energized from a 12 v storage battery, a

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III. Electromagnetic Flow Meter

KENNETH E. JOCHIM, *University of Kansas*

The electromagnetic flow meter is designed to record velocity of blood flow. Special advantages are: (1) the blood vessel need not be opened, (2) its calibration curve is a straight line passing through the origin, and (3) it faithfully records rapid changes in blood flow occurring during each cardiac cycle, thus making it possible to record an instantaneous velocity pulse. The method was first developed by Kolin in this country (1, 2, 5) and independently by Wetterer in Germany (8, 9). The d-c model was subsequently modified as described below, and an a-c model was later developed by Kolin (3, 6).

Principle.—The principle on which operation of the instrument depends is that when an electrical conductor moves across the lines of force of a magnetic field, a potential difference is created in the conductor. If (*a*) the field is uniform, (*b*) the conductor moves in a plane at right angles to the magnetic field and (*c*) the length of the conductor extends at right angles to both field and direction of motion, the resulting potential difference will be directly proportional to field strength, speed of motion of the conductor and length of the conductor within the field; viz.,

of the vessel is kept constant. Two types of sleeves are shown in Figure 2. Type *A* is made of lucite or polystyrene. Size of the lumen of the sleeve and the slot through which the blood vessel is inserted is determined by the diameter of the blood vessel. Two small holes diametrically opposed and oriented at right angles to the axis of the sleeve are provided for receiving short lengths of thick wool yarn soaked in an agar saline mixture and connected to the nonpolarizable electrodes discussed later. The yarn is packed rather tightly in the holes and should project into the lumen a fraction of a millimeter to insure good contact with the vessel wall. Type *B* affords a considerably larger electric contact with the blood vessel. The two halves, of porous baked clay, are held together, but insulated from each other, by a thin piece of bakelite, lucite, celluloid or other insulating material to which they are cemented. The clay pieces, soaked in 0.9 per cent sodium chloride, act as both restraining sleeve and electrodes. Each half has in the top a small well into which the ends of wool yarn may be packed. Clay sleeves of any size are easily molded in two halves from ordinary modeling or potter's clay which, after air drying, is fired at around 1700 F for a few hours. Exact temperature and time of firing must be determined by experience for the particular type of clay used. After firing, the clay halves are cemented in place (Fig. 2, *B*).

Several types of nonpolarizable electrodes have been tried; the most stable operation has been obtained by use of calomel half-cells. They are more bulky than Ag-AgCl electrodes, but with the latter it is difficult to avoid spurious potentials arising probably from variable contact between electrode and vessel wall. The calomel half-cells we use have the form indicated in Figure 2, *C*, although any other convenient shape may be used. Contact between half-cells and blood vessel is made by wool yarns soaked in agar saline. The wicks should be kept as short as possible and prevented from touching any surrounding objects by enclosure in short lengths of thin rubber tubing. Both agar and rubber tubing tend to reduce drying of the wicks to a minimum. From time to time it may be necessary to moisten them with a drop or two of saline.

c) Amplifier.—To record the absolute magnitude of blood flow the d-c component of flow potentials must be amplified. This may be accomplished by three different types of circuits: (1) a direct coupled amplifier, (2) a capacitatively coupled amplifier with mechanical or electronic "chopper" in the input circuit, (3) a circuit whereby the flow potentials may be made to modulate a constant sinusoidal carrier frequency which is then demodulated before recording. We have chosen the direct coupled amplifier described here.

With any direct coupled amplifier the chief problem in design is to eliminate or make negligible zero drift. This has been accomplished by means of the circuit (Fig. 3) modified from a design by Miller (7). It consists of two directly coupled 6SC7 double triodes, coupled through a VR105 gas tube to a 6F8G double triode output tube. The first two stages employ "cathode compensation" for reducing zero drift as described by

central magnetic field strength of approximately 1500 gauss is produced in a 1 cm air gap. The two pole pieces are of soft iron; one is movable so that air gap may be adjusted to size of the blood vessel. This magnet has been used to measure blood flow in such vessels as the carotid artery and inferior vena cava of the dog. *A* is a larger permanent magnet (Alnico V), such as is supplied for use with vacuum tubes of the magnetron type. A field strength of approximately 7500 gauss is available in a 2 cm gap. By means of small soft iron pole pieces, width of the gap may be decreased and correspondingly larger field strengths obtained. A magnet of this type may be used for measuring flows through large tubes which

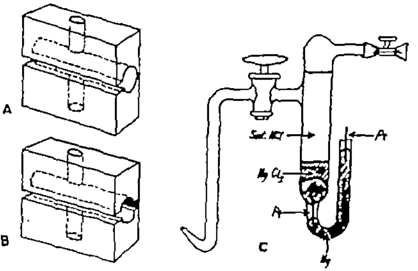

FIG. 2.—*A* and *B*, sleeves for blood vessels; *C*, calomel half-cell nonpolarizable electrode.

form part of a perfusion system such as that employed for heart-lung or isolated heart preparations. Here, of course, space limitations are not so severe and a magnet of this size may be conveniently used.

Such a magnet, when suspended in an inverted position, may also be used to measure flow in the pulmonary artery of a dog with open chest. With careful placement of the magnet and by use of pole pieces of proper size, the pulmonary artery may be placed approximately in the center of the magnetic field.

Electric contact between any part of the magnet and the surrounding tissues should be prevented by coating the magnet with insulating varnish and by providing additional insulation, if necessary, through use of sheets of rubber or celluloid.

b) *Sleeve and electrodes.*—The sleeve has two functions: (1) to hold the electrodes in place on the surface of the blood vessel, and (2) to prevent pulsatile changes in diameter of the vessel. The latter condition is necessary because, since the flow meter records linear velocity of flow, it can be calibrated in terms of volume rate of flow only if the cross-sectional area

Miller. Inclusion of variable inverse feed-back improves stability of operation, minimizes effects of variations in tube characteristics and also provides one means of varying amplifier gain. An attenuator in the input circuit provides further gain control. Both voltage and current outputs are available from the plates and cathodes, respectively, of the last tube. Over-all voltage gain may be varied from 500 to 90,000, and over-all transconductance when using current output may be varied from 0.027 to 5.5 mhos. Both voltage gain and transconductance depend on the value of resistance connected across the two cathodes of the output tubes. For maximal voltage gain these cathodes should be shorted together. For maximal transconductance the galvanometer connected between the two cathodes should have the lowest possible resistance. The frequency characteristic of the amplifier also depends on the value of this resistance between the output cathodes. When resistance is zero (cathodes connected), amplifier response is flat from zero frequency (d-c) to approximately 1000 cycles/sec and rises to a peak at 9500 cycles. When this resistance equals 3000 ohms, response is flat to 7000 cycles/sec and falls off at higher frequencies. Resistance values greater than 3000 ohms further restrict the high frequency range.

Heaters of the tubes are supplied from a 6 v storage battery; plate voltages are obtained from B batteries. The amplifier may be operated from the a-c line if a well regulated and well filtered power supply is used.

It is extremely important that manganin or Advance wire wound resistors be used where indicated. Substitution of other types will result in serious zero drift. The two balancing controls, P_1 and P_2, of helical type, provide critical adjustment of resistance over a wide range and are free from troubles due to poor contact between the moving arm and the resistance wire. It is important also that selector switches and tube sockets make firm positive contacts.

The 6SC7 tube in the first stage should be mounted on a cushioned socket to prevent microphonics and should also be selected for a low inherent noise level. This can be done only by trying various tubes in the first stage and selecting the one which reduces to a minimum the amount of erratic fluctuation (noise) in amplifier output. Stability of operation is improved if all tubes are aged by applying a current to the heaters continuously for 4 or 5 days.

Potentiometer P_3 is General Radio type 314A. Substitution of a cheaper potentiometer of the volume control type is not satisfactory.

Operation of the amplifier requires no particular experience or skill. After an initial warming-up period (preferably about 1 hr), the meter is shunted by closing switch S_7 and connected to the amplifier output by placing switches S_4 and S_5 in appropriate positions. Output is now balanced by means of controls P_1 and P_2, and final balance is attained by further adjustment of P_2 after switch S_7 is opened. Proper setting of P_3 is now found by trial and error. It is properly adjusted when the amplifier

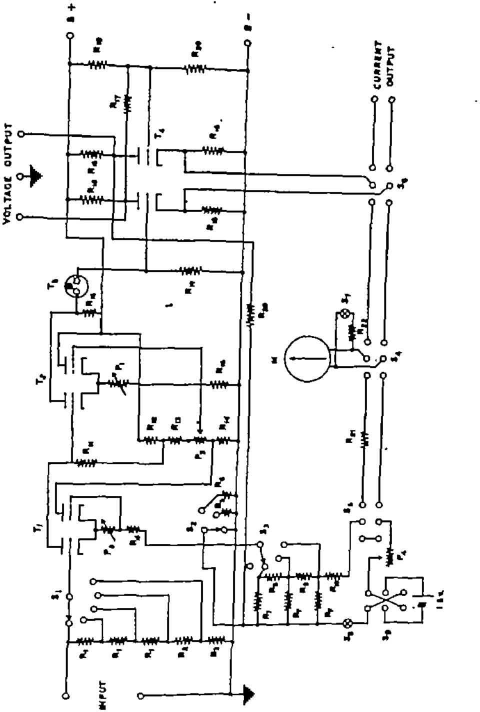

Fig. 3.—Circuit diagram of complete amplifier. Manganin wire wound resistors, 1 w: R_4, 1000 ohms; R_5, 8 ohms; R_6, 72 ohms; R_{11}, 0.5 megacycle; R_{12}, 40,000 ohms; R_{13}, 20,000 ohms; R_{14}, 80,000 ohms. Nichrome wire wound resistors, 1 w: R_1, 0.25 megacycle; R_2, 0.15 megacycle; R_3, 0.10 megacycle; R_7, 10 ohms; R_8, 90 ohms; R_9, 81.8 ohms; R_{10}, 1000 ohms; R_{15}, 50,000 ohms; R_{16}, 0.5 megacycle; R_{18}, 10,000 ohms; R_{22}, 0.11 × resistance of M. Metallized resistors, 1 w: R_{17}, 1 megacycle; R_{19}, 3 megacycles; R_{20}, 0.2 megacycle; R_{21}, 110 ohms. Variable resistors, wire wound: P_1, 10,000 ohms, Helipot; P_2, 10,000 ohms, Helipot; P_3, 1000 ohms, General Radio type 314A; P_4, 500 ohms, volume control type. Switches: S_1, 5-pos. selector; S_2, 3-pos. selector; S_3, 4-pos. selector; S_4, S_5, S_6, S_9, DPDT toggle; S_7, S_8, SPST toggle. Tubes: T_1, T_2, 6SC7; T_3, VR 105–30; T_4, 6F8G. Meter: M 1-0-1 ma.

Miller. Inclusion of variable inverse feed-back improves stability of operation, minimizes effects of variations in tube characteristics and also provides one means of varying amplifier gain. An attenuator in the input circuit provides further gain control. Both voltage and current outputs are available from the plates and cathodes, respectively, of the last tube. Over-all voltage gain may be varied from 500 to 90,000, and over-all transconductance when using current output may be varied from 0.027 to 5.5 mhos. Both voltage gain and transconductance depend on the value of resistance connected across the two cathodes of the output tubes. For maximal voltage gain these cathodes should be shorted together. For maximal transconductance the galvanometer connected between the two cathodes should have the lowest possible resistance. The frequency characteristic of the amplifier also depends on the value of this resistance between the output cathodes. When resistance is zero (cathodes connected), amplifier response is flat from zero frequency (d-c) to approximately 1000 cycles/sec and rises to a peak at 9500 cycles. When this resistance equals 3000 ohms, response is flat to 7000 cycles/sec and falls off at higher frequencies. Resistance values greater than 3000 ohms further restrict the high frequency range.

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is shifted off balance in the same direction regardless of whether P_3 is turned to right or left, i.e., when

$$\frac{dI}{dP_3} = 0,$$

where I is the meter current. Further adjustment of P_3 usually need not be made.

Sensitivity range is selected by means of switch S_2 which, by varying the amount of inverse feed-back, provides maximal voltage gain of 5000, 30,000 or 90,000. Further sensitivity control is provided by the input attenuator, S_1.

The current through the calibration network is standardized by throwing switch *5* to the right and switch *4* to the left with switch *7* open. P_4 is then adjusted until the meter reads exactly 1 ma. A calibrating voltage of 0.1, 1.0 or 10.0 mv is selected by means of switch S_3. The selected calibrating voltage is applied to the amplifier by means of switch S_4.

It is, of course, necessary that all connections in the amplifier be firmly made with rosin-core solder and that the input cable be shielded.

d) Recording galvanometer.—This may be of any type (low internal resistance) which has a frequency response adequate to record the flow pulses in the vessel desired. With a suitable optical system the galvanometer deflection may be recorded on a photokymograph. A commercially available oscillograph (Hathaway, Heiland, General Electric, etc.) may be used.

e) Precautions.—In using the flow meter a number of precautions must be taken to insure satisfactory operation. Size of the sleeve applied to the blood vessel must be selected so as to prevent pulsatile changes in vessel caliber *and at the same time not constrict it sufficiently to interfere with* blood flow. Care must be taken to see that positive contact between electrodes and vessel wall is maintained at all times. No part of the electrodes should be allowed to touch the magnet or any surrounding tissue. The animal must be a sufficient distance from any electrical or magnetic fields or must be adequately shielded from them. The magnet must be so placed that the blood vessel passes through a constant uniform region of the magnetic field. The base line for zero flow is determined by momentarily occluding the blood vessel distal to the sleeve. Any pulsation of the base line during occlusion indicates poor contact between electrodes and vessel wall.

f) Calibration of flow curves.—It may be shown theoretically and experimentally that the calibration curve of the flow meter is a straight line passing through the origin. Consequently, it is necessary to determine *only* one point on the calibration curve. This may be done in vivo as follows: With the blood vessel occluded distal to the sleeve, a measured amount of saline or blood (e.g., 10 ml) is injected with a syringe by introducing the needle into the vessel between the sleeve and the point of occlusion and injecting backward. Resulting deflection of the galvanometer

is recorded with a suitable time marker. The area bounded by this curve and the base line is measured with a planimeter and divided by the scale length of the deflection on the record. This quotient gives the mean height of deflection and is equivalent to a rate of flow given by the total quantity of fluid injected divided by the time taken for injection. Thus one point on the calibration curve is found, and since it passes through the origin, the calibration curve is completely determined.

Critique.—The flow meter is easily applied to any accessible peripheral blood vessel. In vessels close to the heart, however, it is difficult to avoid recording of cardiac action potentials. One way of avoiding this is to cannulate the vessel and thus isolate from the heart the section to which the electrodes are applied.

The instrument has enough flexibility so that its components may be modified in a number of ways to meet individual circumstances as long as the following conditions are satisfied: (*a*) the sleeve must keep the vessel diameter constant, (*b*) the electrodes must be nonpolarizable and must make positive unvarying contact with the vessel wall, (*c*) the magnet must supply a steady uniform magnetic field of sufficient strength to provide flow pulses of adequate size without use of unduly high amplification, (*d*) the amplifying and recording system must have negligible zero drift and must have a frequency-response curve which is flat from zero frequency (d-c) up to the frequency of the highest significant harmonic in the flow pulse to be recorded. It is not known exactly what this upper frequency should be, but it is likely that even for central arterial flow pulses it need not be much above 100 cycles/sec.

NOTE.—This section was reviewed by L. N. Katz.

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IV. Miscellaneous Pulsatile Flow Meters

HAROLD D. GREEN

Techniques other than those already described have been used with varying success for registration of the instantaneous rate of *flow in pulsating* streams. These include: a heated wire, forming one arm of a bridge, passed through a blood vessel and the moment to moment changes in resistance recorded with a string galvanometer connected to appropriate arms of the bridge (2); the bristle tachograph (1), and photographic registration of the rate of movement of a bubble as it is carried along a tube placed immediately in front of a photokymograph (3).

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V. General Comments on Apparatus for Direct Blood Flow Registration

HAROLD D. GREEN

Anticoagulants.—Heparin is the most satisfactory anticoagulant. We use 0.3 ml (3 mg) per kg of body weight initially, followed by 0.05 ml/kg/30 min. Other investigators use as much as 0.5 ml/kg/30 min (p. 192). For perfusion systems we use 0.3 ml of heparin per 100 ml of blood. Heparin appears not to contribute in any way to development of shock in an experimental animal (1). Chlorazol fast pink and related dyes may be used alone or with heparin in a dose of 80 mg/kg. Chlorazol fast pink, however, is slightly toxic in full anticoagulant dosage. Coating of the lining of the apparatus with Silicone* may reduce the amount of anticoagulant required.

Cleaning of apparatus.—Extreme care must be taken to remove all traces of blood from flow meter equipment immediately after use. Failure to do so may result in marked decline in arterial pressure and other changes similar to those seen in cross-transfusion reactions in man when the apparatus is next used. These changes apparently are due in part to products of decomposition of old blood. A very small quantity of blood, such as that residing in the crevices where rubber tubing is attached to metal or glass tubes, seems sufficient to induce the reaction.

* Dry-Film-9987, General Electric Company, 1 River Road, Schenectady 5, N. Y.

Criteria of adequacy of flow meters.—A flow meter should record accurately within ±5 per cent under steady flow conditions. It should also be capable of indicating changes of rate of flow with a lag of not less than 2 or 3 sec when relatively steady flow conditions are occurring or with a lag of not more than 0.01 sec when pulsatile flows are being recorded. The calibration should also remain constant over 1 or more hours for meters used in acute experiments and for days in chronic experiments.

A meter is most conveniently calibrated by passing the fluid to be measured through it at several rates of flow from zero up to the maximum to be anticipated, while recording and simultaneously collecting the outflowing fluid in a graduate or other measuring receptacle for a measured interval of time. All meters should be calibrated at zero flow periodically to be sure that the recording system is not drifting. This is particularly important in velocity flow meters in which calibrations are nonlinear and small deviations near zero indicate proportionately greater flow increments than similar deviations at high flow rates.

Meter lag in following flow changes is important with all meters but particularly in those designed for pulsatile flow. Determination of lag may be performed by two methods. (*a*) In one a stopcock or other device is used to interrupt the flow rhythmically, the moment of interruption and restoration of flow being signaled by some convenient means, depending on the method used to interrupt the flow. When flow is interrupted by an electromagnet, a signal magnet or a mirror attached to the armature of a small relay may be used to signal the opening and closing of the electric clamp. In differential flow meters such as the orifice meter, thermostromuhr and electromagnetic flow meter, the recorder should rise almost instantaneously to maximal rate of flow and should not overshoot or drift after steady flow is maintained. Similarly, return to zero flow on interruption of the flow stream should be as nearly as possible dead beat. In integral flow meters, such as the piston recorder, transition of steady flow to zero flow and back to steady flow should be a sharp angle without overshoot of lag (see reference (2) for examples). (*b*) A second method uses a reciprocal plunger which drives fluid back and forth through the meter. Movements of the plunger can be recorded by attaching to it a mirror which reflects a beam of light into a photokymograph. The plunger can be driven by a suitable mechanical sine wave generator at varying rates of speed and varying strokes. The record should follow such a sine wave with fidelity up to 5–10 strokes/sec for a recorder designed for pulsatile flows and up to 1 cycle/2–4 sec for meters designed for steady flow registration (2). Meters designed for use in pulsatile streams must be checked to be sure that calibrations made under steady flow conditions are applicable to pulsatile flow of the type to be met in the proposed experiment. In practically all arteries this means that the meter must be capable of handling flows which oscillate during one heart cycle from rapid forward flow to backflow frequently equal to 50 per cent of the velocity of forward flow (3).

Loss of pressure occurs in all metering systems. The loss must be kept to an absolute minimum unless an absolutely isolated vascular bed is being studied; otherwise large and unpredictable errors due to collateral circulation artifacts may occur (see p. 218). All meters and connecting cannulae and tubing should therefore be checked for their inherent pressure losses by inserting the inflow cannula in a larger bore T-tube the side-arm of which is connected to a manometer and maintaining the outflow cannula at a constant level. If the manometer is adjusted to zero at zero flow, its reading will give the pressure loss directly, and a second piezometer connection on the outflow is unnecessary. Some energy will be present in the fluid leaving the outflow cannula owing to its velocity, but this is usually negligible. For instance, at 200 ml/min with a cannula of 3 mm internal bore, the kinetic energy of the efflux which would, of course, represent part of the pressure recorded by the upstream manometer would be only 0.8 mm Hg (approximately). The calculation for this factor is:

$$\text{mm Hg} = \frac{V^2}{1.36 \times 2 \times g} = \frac{F^2}{D^4} \times \frac{(4 \times 100)^2}{60^2 \times \pi^2 \times 2 \times 980 \times 1.36} = \frac{F^2}{D^4} \times 1.69 \times 10^{-3}$$

where V equals velocity of efflux in cm/sec; g, gravitational constant = 980; F, flow in ml/min; D, diameter of efflux cannula in mm; 1.36 = factor to convert 1 cm water to 1 mm Hg.

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PERFUSION SYSTEMS

I. Perfusion Systems for Use with Isolated Organs or Regions of the Body

HAROLD D. GREEN

FOR MANY STUDIES blood flow in an organ perfused from the aorta is satisfactory. However, in certain circumstances one may wish perfusion of an isolated organ; in others it is desired to perfuse an organ at pressures other than that in the aorta.

Criteria which must be satisfied in an artificial perfusion system are: the fluid must be maintained at a constant temperature, preferably body temperature; it must be suitably oxygenated; sedimentation of red cells must be prevented; excessive trauma to blood must be avoided.

Ringer's or Locke's solution has been employed in many situations, as in the Läwen-Trendelenburg preparation and the perfused heart and ear. Such solutions avoid the problem of cell sedimentation and are probably suitable for study of vasoconstrictor substances, but sufficient anoxia may exist that local metabolic vasodilation may mask the effect of any dilator substance added to the perfusion fluid.

For blood perfusion we have used small chambers (3), periodically filled with blood from a syringe, the chamber being agitated just before flow measurement and pressure being obtained by regulating air pressure in the chamber.

Various workers (5–7) have used a pump lung system to supply blood to the perfused organs. We have not been too satisfied with this system, feeling that most artificial pumps traumatize the blood sufficiently to release toxic material.

We have recently been using a system whereby a second dog serves as the pump lung system, forcing blood into a reservoir at a rate sufficient to maintain in that reservoir whatever head of pressure is necessary (1). Blood from this reservoir then enters the organ to be perfused in a first dog. To prevent sedimentation in the reservoir, a small constant return from the reservoir flows back into the vein of the donor animal. A diagram of the system is given in Figure 1. An electric contact actuated by a sensitive bellows connected with the air above the fluid in the perfusion reservoir operates an electromagnetic clamp. The clamp admits blood to the reservoir from the donor animal as necessary to maintain pressure at the desired level. It is possible to maintain this perfusion pressure within

2–3 mm over rather wide ranges of blood flow provided a large vessel of the donor animal is cannulated and large bore tubing is used throughout the perfusion system.

For simultaneous perfusion of vascular beds in skin and muscle we have used the arrangement shown in Figure 2.

A highly satisfactory arrangement for perfusion of the whole hindleg is shown in Figure 3 (2). In this preparation the perfusion pressure can be kept quite constant by use of the Lamson and deTurk pressure regulator, i.e., an infusion flask supported at the appropriate height above the animal filled initially with blood from a donor animal and connected to a

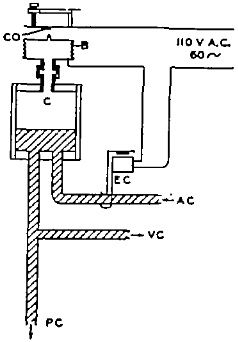

FIG. 1.—Perfusion reservoir. *AC*, cannula inserted in large artery of donor animal; *VC*, return cannula in vein of donor animal; *PC*, cannula in artery of perfused vascular bed; *C*, blood-air chamber of perfusion system; *B*, sensitive metal bellows connected with air chamber of perfusion reservoir; *CO*, adjustable contact for regulating pressure in perfusion system; *EC*, electric clamp, controlled by *B* and *CO*, for regulating flow of blood into *C* from *AC*.

large cannula inserted centrally in a large artery (4). Use of an infusion system to return blood to the animal at approximately the rate at which blood enters the flow meter facilitates maintenance of a constant perfusion pressure. For perfusion of the isolated kidney we have connected the femoral artery and vein of a donor dog to the distal segments of the aorta and vena cava of the kidney dog. Kidney, aorta and vena cava are then dissected free and all branches ligated and finally the aorta and vena cava ligated cephalad from the renal branches (1).

Polythene tubing* has proved highly satisfactory in perfusion systems.

* Obtained from Anchor Plastics Company, 533–41 Canal St., New York 13, N. Y.

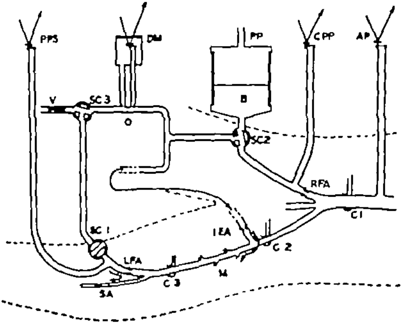

FIG. 2.—Perfusion system for measurement of blood flow in skin and muscle and for collateral circulation studies (p. 219) in dog (indicated by dashed outline). $C_{1,2,3}$, clamps on aorta and femoral artery; *AP*, manometer for recording aortic pressure; *CPP*, manometer for recording pressure in collateral arteries; *PP*, connection to system for regulating air pressure (similar to that in Fig. 1); *B*, blood reservoir; $SC_{1,2,3}$, stopcocks; *O*, orifice meter; *DM*, differential manometer (p. 105); *PPS*, manometer for recording perfusion pressure (blood flow in skin); *V*, viscosimeter; *LFA*, cannula in left femoral artery; *IEA*, cannula in inferior epigastric artery; *M*, branches of femoral artory going to muscle; *SA*, saphenous artery (supplying principally skin). Long arrow and dotted lines indicate another orifice meter and differential manometer used for measuring blood flow in muscle (omitted to simplify diagram).

Systems that have been developed for use with the isolated ear are given on page 123, for the kidney on page 193, for the brain on page 204, and the Läwen-Trendelenburg preparation on page 129. Other perfusion systems have been described by Bruner and Schmidt (1a), Fleisch (1b), Geiger and Magnes (2a), Heubner and Mancke (3a) and Paff *et al.* (52).

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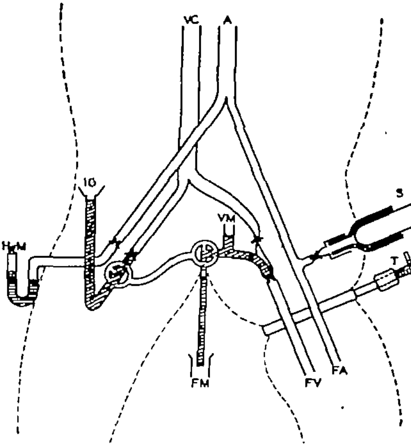

FIG. 8.—Perfusion system for measuring blood flow in hindleg of dog (indicated by dashed outline). *A*, aorta; *VC*, vena cava; *FA*, femoral artery; *FV*, femoral vein; *S*, tuberculin syringe for injecting drugs, etc.; *T*, tourniquet; *VM*, venous pressure manometer; *FM*, venous outflow meter (p. 69); *IG*, infusion system for infusing blood at rate approximating that at which blood is collected in *FM* during flow measurement; *HgM*, mercury manometer for recording perfusion pressure. Shadowed areas indicate path of blood flow during flow measurement. When flow measurements are not desired, venous return can be directed from left into right femoral vein by rotating stopcocks.

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II. Perfusion of Rabbit's Ear for Study of Vasoconstrictor Substances

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Perfusion of the isolated rabbit's ear is a reliable procedure in study and assay of vasoconstrictor substances such as serum vasoconstrictor, angiotonin and some pressor amines.

APPARATUS

There has been little modification of the original apparatus (2). It (Fig. 1) consists basically of a constant temperature box in which the ear is placed, with arrangements for circulating warmed perfusion fluid under pulsatile pressure.

The incubator box (1 cu ft inside), insulated with asbestos and lined with copper, is heated by a 100 w lamp controlled by a de Khotinsky thermoregulator. Under the lamp a pan of water, covered with wire gauze on which surgical gauze is spread, acts as humidifier.

A complete circuit (Fig. 1) of the perfusion fluid through the apparatus is provided for. Wherever possible, connecting tubing is of glass or firm plastic to decrease distensibility. The fluid begins the circuit in a 1 liter aspirator bottle, *A*, from which it passes through a flap valve, *B*, to a heating unit, *C*. The last consists of a glass tube 2 × 13 in. filled with water which contains a 100 w immersion heater of nichrome wire in a monel metal tube surrounded by a glass coil through which perfusion fluid flows. Rubber stoppers secured by bolted disks hold the ends of the tube. Circulation and temperature control is provided by a pump and a thermoregulator, consisting of two round-bottom glass tubes, *D* and *E*, $1^1/_4$ in. in diameter, connected with the heating unit by outlets. Tube *D* is $9^1/_4$ in. high and contains a mercury thermoregulator which activates the heater through a 110 v relay. Tube *E*, $8^1/_2$ in. high, has a piston for pumping the fluid from the heater around the thermoregulator. The piston is attached eccentrically to a 3 in. wheel driven by a small motor. Speed is reduced to about 2 rpm by means of two reducing pulley systems and a worm gear.

The perfusing medium is led from the coil to a small glass chamber, *F*, clamped inside the box. A thermometer is inserted to measure temperature of the fluid. One arm of the chamber is the inlet tube from the heater; the second leads perfusion fluid into the cannula, and the third is an outlet tube attached by rubber tubing which leads excess fluid back to the aspirator bottle. Inserted in this return line is a 1-way glass valve, *G* (Fig. 2, *B*). The rubber tubing ends in one arm of a T-tube. The side-arm of the T-tube goes into a rubber stopper fitted with a flap valve (Fig. 2, *A*). A 50 ml syringe can be attached to one arm of the tube for flushing or filling the circuit.

water. When it is to be used, more distilled water is pulled through the conduits by the syringe, and finally phenol red is added to the contents of the syringe to establish the fact that all alkali has been flushed away. From time to time, bubbles of air may be pulled through the tubing to aid in dislodgment of particles. The cannula is kept clean by storing in weak alkali.

PERFUSION FLUIDS

1. *Modified Ringer's solution.*—The most important factor controlling sensitivity of the preparation is the perfusing medium and, in particular, the ratio of the various ions. Phosphate ion increases sensitivity and calcium ion is essential for adequate responsiveness. However, concentration of calcium ion must be such that phosphate will not be precipitated and citrated or oxalated plasma will not clot when injected into the ear vessels. It is possible that different perfusing media will be used for study of different vasoconstrictors to achieve maximal effectiveness. We now use the medium given in Table 1 for study of serum vasoconstrictor.

TABLE 1.—ROUTINE PERFUSING SOLUTION

SUBSTANCE	CONCENTRATION
Phosphate buffer pH 7.4	0.01M
NaCl	8.2 g/l
KCl	0.84 g/l
$MgCl_2 \cdot 6H_2O$	0.06 g/l
$CaCl_2 \cdot 2H_2O$	0.04 g/l
Glucose	1.0 g/l
$NaHCO_3$	0.2–0.5 g/l

It is prepared by diluting 100 ml of 10 times concentrated stock solution containing all the salts except $NaHCO_3$ and phosphate almost to 1 liter. To this is added 10 ml of phosphate buffer containing 4 parts of 1M K_2HPO_4 to 1 part of 1M KH_2PO_4, the glucose and $NaHCO_3$, and the solution is made up to 1 liter. If free chlorine is present in the distilled water, it is necessary to redistill it from alkaline permanganate.

2. *Plasma.*—For study of certain vasoconstrictors (e.g., the vasoconstrictor in hypertensive patients) it is advisable to perfuse the ear with plasma. In obtaining the blood great care must be exercised that not even the smallest clot form. Citrated blood is perfectly satisfactory. The syringe is wet with citrate and 0.4 ml of 10 per cent sodium citrate used for each 10 ml of blood. After centrifugation the plasma is diluted with 3 volumes of Ringer's solution. The "preheparinized plasma" used by Landis *et al.* (1) can also be employed, but is more expensive and seems to add nothing to the reliability of the method.

PROCEDURE

Preparation of ear.—The ear of an unanesthetized New Zealand white rabbit is clamped at the base with an intestinal clamp and severed just

distal to the clamp with one swift stroke of a sharp scalpel. The skin is slit on either side of the central artery for about $^3/_4$ in. and the skin flap pulled back and cut off. The artery is carefully dissected out and held steady with a small hemostat attached to the end. A small V incision is made into the artery close to the hemostat and the glass cannula inserted and tied with silk. Warm Ringer's solution is used to wash out the remaining blood and insure patency of the cut ends of the veins. The whole procedure can be completed in 2 or 3 min. Although speed is desirable, it is not essential, since ears difficult to cannulate either because of inexperience of the operator or size of the artery have proved satisfactory.

The ear is placed on the draining plate, secured with adhesive tape, and a piece of string placed against the cut surface to lead the perfusate into the collecting funnel. The cannula is connected and the perfusion solution (37 C) allowed to flow through the ear. Position of the ear must be correct, i.e., the artery straight, but not tense or twisted, and the system free of air bubbles. When perfusion is first started it may be necessary to use pressure of 40–50 mm Hg, but after a few minutes systolic pressure is usually about 10 mm for drop rates of 20/min when modified Ringer's solution (Table 1) is employed.

Injection of vasoconstrictors.—Injections usually of 0.1 or 0.2 ml are made through the serum bottle stopper with a 0.5 or 1 ml tuberculin syringe fitted with a $^1/_2$ in. 27 gauge needle. Before injection the drop rate must be adjusted to the desired constant value by controlling air pressure, and the previous constriction must be completely over; i.e., drop rate must have reached a constant level. This may be 90–110 per cent of the rate prevailing before the previous injection. Care is necessary since prolonged constriction may be followed by momentary dilation. After each of the first two or three constrictions are over, drop rate may be faster and pressure may need to be lowered to retain the control drop rate.

It has not been found necessary to observe the precautions taken by Landis *et al.* (1) to prevent cooling of the ears while opening the door for the few seconds required to make an injection. It is impossible in our preparation to observe any constriction of the kind they describe, even when the door is opened purposely for prolonged periods.

Sensitivity of ear vessels.—Usefulness of the ear as an instrument for assay depends on its sensitivity. This is greatly enhanced by injection of fresh serum or adrenalin. Routinely, the first injection consists of 0.1 ml of fresh cattle or dog serum plus 0.1 ml of 2×10^{-4} adrenalin. The serum may be kept frozen and thawed immediately before use. Following this, 0.2 ml of serum diluted 1:10 with Ringer's solution should give at least a 5 min constriction at a control drop rate of 20/min. If the ear is insufficiently sensitive, undiluted serum or adrenalin is reinjected. Repeated constrictions increase sensitivity and seem to prevent formation of edema. The latter, however, does not decrease usefulness of the ear. If

water. When it is to be used, more distilled water is pulled through the conduits by the syringe, and finally phenol red is added to the contents of the syringe to establish the fact that all alkali has been flushed away. From time to time, bubbles of air may be pulled through the tubing to aid in dislodgment of particles. The cannula is kept clean by storing in weak alkali.

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III. Läwen-Trendelenburg Preparation for Perfusion of Hindleg of Toad

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PROCEDURE

a) *Material.*—Marriotte flask; glass cannula or round tip needle for the aorta and glass cannula for the abdominal vein; if any substances are to be tested in small amount, a T-tube with a needle and a syringe adapted at its free branch (Fig. 1, *V*, *T–T*); rubber tubing for connections between aortic cannula, T-tube and Marriotte flask; cork board for toad; pins. Perfusion liquid: any adequate frog perfusion liquid, such as Hülse's (2): NaCl 140 g, KCl 10 g, sodium citrate 100 g, distilled water to 1000 ml (keep in refrigerator to the moment of use and dilute 20 times before using).

b) *Technique.*—The toad is fastened on its back on a frog board after its medulla and brain have been destroyed with an appropriate needle. The skin and abdominal muscles are cut transversely at the level of the xiphoid process (Fig. 1, *I*, *TS*). This incision is extended caudally as two lateral incisions approximately 1 cm from the midline (*I*, *LI*). The abdominal vein (*II*, *III*, *AVL*) is tied, severed between this tie and the liver, and the abdominal flap is reflected caudally (*II*, *III*, *IV*, *AF*). The peritoneum in the posterior abdominal wall and the peritoneum from the bladder to the abdominal wall (*II*, *III*, *P*) is cut at both sides, exposing the retroperitoneal organs. The aorta is tied and cut as high as possible (*II*, *AL*). The vein (*III*, *V*) which passes from the bladder to the abdominal vein in a fold of the peritoneum (*III*, *PF*), clearly seen when the abdominal flap is turned over and the bladder pulled toward the head, is tied and cut between the ligature and the bladder (*III*, *B*).

After the bladder is freed a mass ligature (*IV*, *ML*), passed behind the bladder, rectum and both renal portal veins (*VI*, *RP*), is tied. The bladder and intestines are severed proximal to the tie and all the abdominal organs removed, exposing the aorta. A cannula connected with the Marriotte flask (*IV*, *V*, *MF*) is inserted in the aorta just distal to the ligature (*IV*, *AL*) and tied in place just proximal to the bifurcation

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the ear is to be idle for a protracted period, it is best to inject serum and reduce perfusion pressure so that prolonged constriction results. An ear used successfully with increasing sensitivity during the day can be kept overnight in the refrigerator and used for a number of hours the next day.

Calculation of degree of vasoconstriction.—We have found it convenient to report the degree of vasoconstriction in terms of per cent constriction of a certain duration. The number of drops is counted from the time vasoconstriction is first observed until it is virtually over (A). This value is subtracted from the number of drops there would have been had no constriction occurred, i.e., time in minutes multiplied by initial constant drop rate (T). The value $100(T-A)$ divided by T is the per cent constriction. The value $T-A$ is also the value of the area under the curve (time against number of drops/min) plotted by Landis *et al.* (1).

Unfortunately, there is no simple mathematical expression combining initial drop rate, duration of constriction and degree of constriction. The slower the drop rate and the lower the pressure, the greater the duration and degree of constriction. In practice, when vasoconstriction caused by two solutions is to be compared, it is advisable to keep the initial drop rate constant, thereby eliminating one variable. An example of the assay follows.

TABLE 2.—EXAMPLE OF ASSAY OF SERUM VASOCONSTRICTOR
(Initial drop rate 20/min)

MIN SINCE BEGINNING OF CONSTRICTION	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$	4
Solution Injected	Total No. of Drops						
Control	6+	9	12+	16	21	27	$34\frac{1}{2}$
Unknown diluted 1:200	6	$8\frac{1}{2}$	11−	$13\frac{1}{2}$	16	20+	25−
Unknown diluted 1:300	7	$10\frac{1}{2}$	13	$19\frac{1}{2}$	$25\frac{1}{2}$	33	$41\frac{1}{2}$
Control	6	9	12	16	21+	29+	33
Unknown diluted 1:250	6−	9−	12	16−	21	27	34

Example: Since degree of response to injection of a substance varies from ear to ear and from time to time in the same ear, all solutions to be assayed must be compared with a control solution, i.e., hypertensive plasma with normal plasma and serum vasoconstrictor at various degrees of purification with a stable, although impure, preparation that can be kept over long periods. Use of very dilute solutions of adrenalin is impracticable because of its instability in slightly alkaline solutions. In practice, dilution of control serum vasoconstrictor is selected which will give constriction of 3–4 min duration at a basal constant drop rate. This solution is injected alternately with varying dilutions of the unknown solution until control and unknown give identical constrictions. Drop rate is counted at $\frac{1}{2}$ min intervals beginning at the first indication that constriction has started. With this procedure, variations of 25 per cent in concentration of serum vasoconstrictor can be determined.

NOTE.—This section was reviewed by Eugene M. Landis.

INDIRECT METHODS FOR REGIONAL BLOOD FLOW

I. Microscopic Observations of Circulation in Rat Mesoappendix and Dog Omentum: Use in Study of Vasotropic Substances

BENJAMIN W. ZWEIFACH, *Cornell University*

Direct visualization of the capillary bed offers a means for studying reactions of the several components of the peripheral circulation under carefully controlled conditions. Significance of the observations depends largely on precautions taken to maintain the exposed tissues under as normal conditions as possible. Local tissue changes seriously interfere with the precisely balanced, spontaneous vascular phenomena of local and systemic origin which regulate peripheral distribution of blood (1).

In the mammal some form of anesthesia is routinely used to immobilize the animal for experimental procedures. Specific effects on different functions of the organism which each anesthetic agent introduces, in addition to those attributable to the over-all anesthetic action, require careful consideration in studies on peripheral circulation, where specific drug action of various anesthetic agents profoundly alters vascular reactivity to chemical substances and physiologic stimuli (7). Depth of anesthesia is also important. It is preferable in experiments on the peripheral vascular apparatus to maintain the animal in the upper first plane of surgical anesthesia. Under such conditions corneal reflexes are brisk and respiratory movements of the thorax relatively unaffected. For small mammals such as the rat, sodium pentobarbital or seconal has been satisfactory. In young rats (100–125 g), average anesthetic dose administered intramuscularly is 3–3.5 mg/100 g body weight and in older rats, 3.5–4.0 mg/100 g body weight. For the dog, 30 mg/kg of sodium pentobarbital is injected intravenously, the final 50–75 mg being administered slowly while reflexes and respiration are checked repeatedly. When dog omentum is to be studied, it is possible to use morphine sulfate (6–8 mg/kg) intravenously, with local infiltration of 1 per cent procaine to facilitate the abdominal surgery.

In selection of a tissue for microscopic study two requisites should be met: (*a*) preparation of the tissue should involve an absolute minimum of trauma, and (*b*) the vessels in the observed field should lie sufficiently in one plane so that the extent of their course from arteriole to venule

(*IV*, *AC*). An incision is made in the abdominal vein near the tie and perfusion is begun. After the perfusion liquid draining out becomes clear a glass cannula is inserted in the abdominal vein (*IV*, *AVC*) and the drops issuing from it are counted or recorded (see p. 72). Perfusion pressure (*V–PP*) must not be more than 16–20 cm of water, which gives an outflow of 40–60 drops/min. Any obstacle to outflow on the venous side on either veins or cannula must be avoided. Sensitivity increases for several hours, hence it is better to wait 2 or 3 hr before making a trial. The mechanical effect of the volume of liquid injected in the inflow tube is immediate and may be discarded with a control sample.

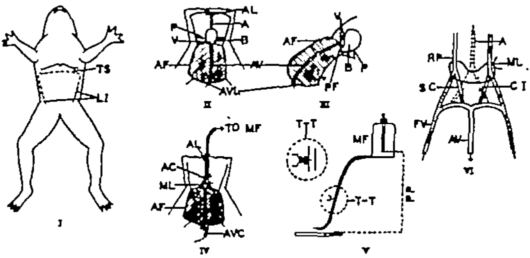

Fig. 1.—Perfusion of hindleg of toad.

The schema of the arterial and venous system of the circulation of the hindlegs (*VI*) shows how perfusion is established. Liquid infused by way of the aorta (*A*) circulates through the common iliacs (*CI*) to the hindlegs and returns by way of the femoral (*FV*) and sciatic (*SC*) veins to the abdominal vein (*AV*).

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INDIRECT METHODS FOR REGIONAL BLOOD FLOW

I. Microscopic Observations of Circulation in Rat Mesoappendix and Dog Omentum: Use in Study of Vasotropic Substances

BENJAMIN W. ZWEIFACH, *Cornell University*

DIRECT VISUALIZATION of the capillary bed offers a means for studying reactions of the several components of the peripheral circulation under carefully controlled conditions. Significance of the observations depends largely on precautions taken to maintain the exposed tissues under as normal conditions as possible. Local tissue changes seriously interfere with the precisely balanced, spontaneous vascular phenomena of local and systemic origin which regulate peripheral distribution of blood (1).

In the mammal some form of anesthesia is routinely used to immobilize the animal for experimental procedures. Specific effects on different functions of the organism which each anesthetic agent introduces, in addition to those attributable to the over-all anesthetic action, require careful consideration in studies on peripheral circulation, where specific drug action of various anesthetic agents profoundly alters vascular reactivity to chemical substances and physiologic stimuli (7). Depth of anesthesia is also important. It is preferable in experiments on the peripheral vascular apparatus to maintain the animal in the upper first plane of surgical anesthesia. Under such conditions corneal reflexes are brisk and respiratory movements of the thorax relatively unaffected. For small mammals such as the rat, sodium pentobarbital or seconal has been satisfactory. In young rats (100–125 g), average anesthetic dose administered intramuscularly is 3–3.5 mg/100 g body weight and in older rats, 3.5–4.0 mg/100 g body weight. For the dog, 30 mg/kg of sodium pentobarbital is injected intravenously, the final 50–75 mg being administered slowly while reflexes and respiration are checked repeatedly. When dog omentum is to be studied, it is possible to use morphine sulfate (6–8 mg/kg) intravenously, with local infiltration of 1 per cent procaine to facilitate the abdominal surgery.

In selection of a tissue for microscopic study two requisites should be met: (*a*) preparation of the tissue should involve an absolute minimum of trauma, and (*b*) the vessels in the observed field should lie sufficiently in one plane so that the extent of their course from arteriole to venule

can be kept under continuous observation. Data on single isolated capillary loops or small sections of a vessel are highly unsatisfactory and frequently misleading.

Essential to understanding of the functional activity of the peripheral vascular apparatus is recognition that the architectural pattern of the capillary bed varies in different regions of the body according to the

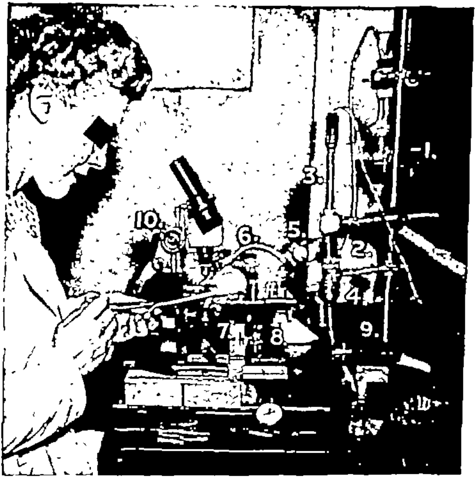

FIG. 1.—Apparatus for study of circulation of mesoappendix of rat. Anesthetized animal on rat board, *7*, attached to 3-way movement, *8*, leaving tail accessible for intravenous injections. *1*, 1 ml Florence flask; *2*, 50 ml Erlenmeyer flask; *3*, thermoregulator; *4*, nichrome heating element; *5*, screw clamp; *6*, 17 gauge hypodermic needle; *9*, lamp; *10*, microscope.

functions performed (2). The basic function of the capillary system in tissues such as the mesentery or skeletal muscle is nutritive. In certain organs, because of specialized functions, atypical arrangements of the vascular bed exist and mask the basic nutritive pattern, as in the liver spleen and kidney. It is also true for the vessels of the skin which, readily accessible, are frequently used for microscopic study of the lation. However, identification of the vascular components in often complicated by anastomoses between superficial

more deeply placed venous plexuses. A further disadvantage is the difficulty of applying substances directly to the cutaneous vessels, a procedure which serves to locate and to quantitate changes in their functional reactivity. Satisfactory regions for microscopic study of the peripheral circulation in the skin are the interdigital web of the rat, ear of the mouse or rabbit and corneoscleral junction of the rabbit eye.

Of visceral structures sufficiently transparent for detailed study of the peripheral blood vessels, the mesentery and omentum are the most useful. For routine wotk the so-called mesoappendix or mesocecum of the rat, a triangular flap of mesentery lying between the cecum and the terminal portion of the ileum, is readily prepared for microscopic study (5). In the dog and cat, the omentum is most suitable (6). The intestinal mesentery was avoided because of continued and pronounced peristalsis and difficulty in preventing edema of the exposed loop.

PROCEDURE IN RAT MESOAPPENDIX

Animals.—A Wistar strain of rats, 40–50 days old, weighing 100–125 g, is satisfactory.

Microscope equipment.—An ordinary laboratory microscope; preferably binocular, with the stage removed is used. A 15× ocular in combination with a 4×–10× objective gives adequate magnification. The upper lens of the substage condenser is removed so that the light is focused about 1 cm above the level of the stage. A low voltage lamp (6 v, 3–5 amp) is the source of light (Fig. 1, *9*).

Rat board.—The rat is mounted on a 6 × 4 in. board attached to a 3-way rack and pinion manipulator movement (Fig. 1, *7*). One corner of the board is cut out to include a glass plate on which the tissue to be transilluminated is placed. The exteriorized tissue is draped over a glass horseshoe made from strips of glass about $^{1}/_{8}$ in. wide, the width of the U being $^{1}/_{4}$ in. and the length of the sides $^{3}/_{4}$ in.

Drip mechanism.—The exteriorized tissue is kept moist and warm by irrigation with Ringer's solution containing 1 per cent gelatin. Powdered ash-free gelatin is dissolved in Ringer's solution by warming the mixture. Before use the mixture is filtered through coarse filter paper under suction. Several drops of a 1 per cent solution of phenol red is then added, giving a yellow color. Sodium bicarbonate powder is carefully added until the color changes to the pink color range corresponding to pH of 7.0–7.2. A 1 liter Florence flask serving as a reservoir in turn is connected to a 50 ml Erlenmeyer flask, having three sidearms, one for inflow of the Ringer-gelatin mixture, one for outflow and one for withdrawal of fluid from the flask. The flask is heated by a nichrome heating element. An immersion mercury thermoregulator connected to a suitable relay maintains temperature of the solution. A screw clamp on the rubber tubing leading from the Erlenmeyer flask to a 17 gauge needle, freely movable on a ball and socket joint, controls rate of outflow onto the exposed tissue.

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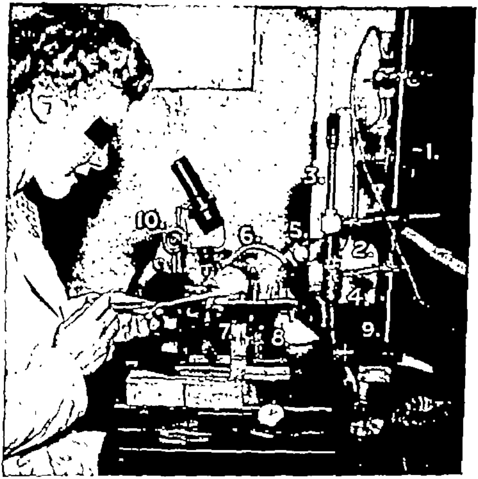
Essential to understanding of the functional activity of the peripheral vascular apparatus is recognition that the architectural pattern of the capillary bed varies in different regions of the body according to the



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To use the epinephrine response as a quantitative index of changes in functional state of the capillary bed, observations must be confined to the same selected vessel throughout. This is necessary, since the threshold response to epinephrine varies with different arterioles in the same tissue.

Epinephrine solution.—A stock solution of 1:100,000 is prepared. Dilutions are made with Ringer-gelatin medium in a 1.0 ml tuberculin syringe and applied through a 17 gauge needle to the surface of the exposed mesoappendix. An important factor is temperature of the Ringer-gelatin solution used to dilute the epinephrine. Cold Ringer's solution applied to the mesentery will itself cause vasoconstriction. The diluting medium should therefore be kept at body temperature on a warming table or in a water bath. The stock solution of epinephrine should not be warmed, since the amounts used are small in comparison with the volume of diluting solution. The routine is first to use a dilution of epinephrine which is on the average just below that required to cause a visible constrictor effect; 1 part in 6,000,000 has been found suitable for this purpose.

Determination of epinephrine threshold.—The drip bathing the mesentery is turned to one side and 3–4 drops of a 1:6,000,000 concentration of epinephrine applied to the tissue surface. The test should be repeated with increasing or decreasing concentrations of epinephrine until a dilution is reached which causes a threshold constrictor response. The type of reaction to be looked for is moderate narrowing of the terminal arterioles and precapillaries, just sufficient to slow blood flow through the capillaries and tributary venules in 15–20 sec. This minimal effective or threshold concentration should be rechecked several times. Between each epinephrine test the drip is restored to the tissues. Tests should not be made more often than at 3 min intervals, since repeated application of epinephrine sensitizes the vessels to the drug.

Criteria for normal state of preparation.—Most satisfactory results were obtained with preparations meeting the following criteria.

1. When 3–4 drops of epinephrine is applied to the surface of the mesentery, terminal arterioles and precapillaries should respond to concentrations between 1:1,000,000 and 1:5,000,000. In several thousand rats, prepared as described, a satisfactory reaction has been obtained with concentrations of 1:2,000,000–1:4,000,000. Preparations which require threshold concentrations greater than 1:1,000,000 are abnormal and should be discarded. Occasional animals show an asphyxic type of response owing to presence of mucus in the trachea, mild pneumonia or poor respiration following excessive anesthesia. Such preparations will show an abnormally heightened epinephrine reaction and should also be discarded.

2. Blood flow through the capillary bed should be intermittent. A continuous, rapid flow through all of the capillary vessels indicates abnormal dilatation of the larger blood vessels feeding the tissues.

Drip should be adjusted so that 50–60 drops/min maintain the temperature, estimated by an ordinary mercury thermometer, at 37–38 C.

Temperature.—Accuracy of the test depends on maintenance of a constant level of vascular reactivity throughout. Fluctuations in temperature of the fluid bathing the tissue alone will cause changes in response of the peripheral blood vessels. Temperatures of 30 C or below make the vessels hyper-reactive; those above 38 C dilate the vessels and make them less responsive to stimuli. To avoid fluctuations in temperature of the drip fluid, it is important to maintain the room temperature at a given level, preferably about 75 F, and to avoid drafts or frequent opening and closing of doors leading to the room.

Anesthesia.—Intramuscular injection of 3–3.5 mg of sodium pentobarbital or seconal per 100 g of body weight produces light surgical anesthesia. Owing to considerable individual variation, an attempt should be made to produce comparable planes of anesthesia in the different animals. The rat, properly anesthetized, should not react to painful cutaneous stimuli. Overanesthetization will depress completely the reaction of terminal vascular bed to vasoconstrictor agents.

Drip solution.—Probably the most important feature for maintaining normal conditions, aside from temperature, is continuous irrigation of the exposed tissue with a colloid-containing salt solution (1). The effectiveness of adding gelatin to the perfusate is shown by the fact that with Ringer's solution alone it was not possible to prevent edema or petechial hemorrhages or to maintain the preparation for more than 1 hr without progressive loss of responsiveness of the vascular components. With 1 per cent ash-free gelatin in Ringer's solution, the normal state could be maintained as long as 3–4 hr.

Exposure of tissue.—An incision about 1 in. long is made in the lower right quadrant of the abdomen and the cecum is exteriorized by gentle pressure on the abdominal wall. It may be necessary to bring the cecum through the incision with rubberized forceps, but at no time should gut be stretched or pulled. Excessive handling causes the vessels to become unduly dilated and unresponsive. The exposed tissue is completely covered by cotton moistened with warm Ringer-gelatin solution. Before the rat is transferred to the board, temperature of the drip is given a final check. A glass horseshoe is gently inserted between the cecum and the terminal end of the ileum so that the triangular flap of mesentery is draped over the glass support without stretching of the tissue. The exposed portion of gut is covered with moist cotton and the Ringer-gelatin drip is directed onto the mesentery. The preparation is now ready for microscopic study.

Selection of vessel.—To permit direct comparison of results in different animals, vessels of the same type should be selected for reactivity studies. The observer should become familiar with the characteristic appearance and distribution of the terminal arterioles (about 15–20 μ in diameter) and their type of branching, especially of the precapillary sphincters.

components of the vascular tree to blood-borne principles. Their reaction to epinephrine topically applied provides a criterion for semiquantitative assay of principles whose action is primarily on the peripheral blood vessels. Under carefully controlled conditions response of the peripheral vessels to epinephrine remains fairly constant. Consequently, any deviation from the control type of response to epinephrine which follows systemic injection of test substances could be used as an indication of their vasotropic activity.

Administration of test material.—The skin of the tail is rubbed lightly with xylol to increase visualization of the tail veins and about 0.5 ml of the agent is injected with a 27 gauge needle. Introduction of greater volumes is unsatisfactory. The injection should be made slowly and with care that blood can be drawn back into the syringe at the end of the injection. The pH of solutions other than blood should be carefully controlled. Whenever possible with chemical preparations, extraneous substances which produce side-reactions, e.g., potassium and ammonium sulfate, should be removed by exhaustive dialysis.

Responses.—The samples are classified under three categories.

1. Neutral. Many samples have no demonstrable effect on capillary vessels. For example, normal blood plasma or serum causes only a very transient speeding of the capillary flow.

2. Vasoexcitor. In this category are placed samples which enhance the reactivity of the terminal arterioles to epinephrine. After injection of such a substance complete cessation of capillary blood flow is usually obtained with threshold concentrations of epinephrine which normally produce only partial slowing of flow. The minimal effective concentration of epinephrine with vasoexcitor agents is frequently as low as 1:20,000,000–1:30,000,000, a 5–10 fold change from control values.

3. Vasodepressor. Many samples depress epinephrine reactivity or completely abolish it. Thus, the normally effective threshold concentration of epinephrine, when topically applied, produces no visible change in the capillary bed. Occasionally, with some samples a moderate response to epinephrine persists, the reaction to epinephrine appearing much slower than normally and producing only transient slowing in capillary blood flow. Such samples are designated mild depressors.

The vasotropic activity of different samples is compared by noting duration of the vascular effect obtained. Different vasodepressor samples depress epinephrine reactivity for 4–25 min and are graded accordingly. A more quantitative evaluation of the samples can be obtained by ascertaining the precise concentration of epinephrine needed to produce a threshold type of constrictor response. These values can then be plotted on a time concentration curve.

On the average, only two samples are tested on a given rat. When the initial injection produces pronounced vascular effects the animal is discarded after only a single test. No tests are made on animals which have been on the rat board more than 50–60 min.

3. There should be a minimum of leukocytic sticking or diapedesis in the venules.

4. No capillary stasis or stagnation of blood should be present. Petechial capillary hemorrhages indicate undue handling of the preparation or an abnormally high drip temperature.

5. The larger arteries and veins in the mesoappendix are 100–250 μ in diameter. Under normal conditions the artery is usually somewhat narrower than its accompanying vein.

PROCEDURE IN DOG OMENTUM

To prepare the omentum, a transverse incision is made on the left side just below the last rib with the dog lying on its back. A portion of the omentum is withdrawn and inserted in a thin rubber sheath, the edges of which are sewn to the peritoneal margins of the wound. When ready for study the outer end of the sheath is fastened to the side of a specially prepared moist chamber on a stage under a microscope. A small part of the omentum, protruding from the sheath, is now gently spread over a horseshoe-shaped ribbon of glass which rests on the bottom of the chamber. The excess folds of omentum around the horseshoe lie on and are covered with cotton. All exposed tissue beyond the sheath is continuously irrigated with a drip of Ringer-gelatin solution kept at body temperature. The observed portion of the omentum must be kept uncovered because of its tendency to exude a tenacious opaque material when in contact with solid surfaces.

Every possible precaution is taken to minimize trauma while exposing the tissue. Not only should there be good capillary flow, but the vessels should retain their normal responsiveness to stimuli. A routine test for this is topical application of a few drops of histamine or epinephrine. Injury to a tissue tends to dilate the vessels so that the dilator response to histamine is minimized and also eliminates specific differences in constrictor responses of the various vessels to epinephrine. For dog omentum, under pentobarbital anesthesia, the minimal effective concentration of epinephrine is 1:4,000,000–1:5,000,000 and, of histamine, approximately 1:1000. The dilutions are always made in Ringer-gelatin solution. During topical application, the Ringer-gelatin drip is stopped. Four to 5 drops of a given reagent is then deposited while the tissue is under view through the microscope. A reaction is looked for in the first 20–30 sec and is regarded as negative if no change occurs. At least 3–4 min is allowed to elapse between separate tests.

RAT MESOAPPENDIX TEST FOR VASOTROPIC AGENTS

The reactions in the peripheral vessels are sufficiently consistent and selective to serve as specific criteria for detection and assay of blood-borne vasotropic substances (3, 4). The rat mesoappendix is highly satisfactory for this type of assay. Especially suitable are the terminal arterioles and precapillaries, since they represent the most responsive

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II. Transparent Chamber Technique

HIRAM E. ESSEX, *Mayo Foundation*

Through the work of Sandison (14) and Clark and his students (7) transparent chambers placed in the ears or other parts of the body of animals have become standard in a variety of investigations. Algire (4) devised a chamber for use in study of tumor growth in mice. One of the simplest chambers to prepare is the modification by Sanders and associates (13) of that of Clark. My colleagues and I have used this type of chamber for several years with satisfaction.

APPARATUS

a) *Chambers.*—Of the various plastics used in fashioning transparent chambers, lucite is the most satisfactory from all points of view. The chambers can be made in any well equipped shop. Essential tools and materials follow.

EQUIPMENT	MATERIALS
1. Metal-turning lathe	1. Lucite bar 25 mm in diameter*
a) beveling tool	2. Sheets of mica 0.1 mm thick
b) narrow cutting tool	3. Kodaloid 70 μ thick
c) drill, diameter 7 mm	4. Copal glue
d) millimeter caliper	5. Ethyl acetate (pure solution)
2. Emery wheel	6. Threaded rods with nuts
3. Drill, 1 mm	7. Silver polish
4. File, fine	8. Polishing silk (stocking satisfactory)
5. Screw clamp	
6. Emery paper, very fine	
7. Glass tubing—drawn to capillary	
8. Dissecting microscope (desirable)	

Procedures used by our technician, W. A. Meeker, in making the chambers follow.

1. The lucite bar is centered in the lathe and a hole 7 mm in diameter drilled in the center to a depth sufficient for making several disks 1.5

* Lucite obtained from Du Pont. Brass or sterling silver threaded rods 18 mm long, 1 mm in diameter with 128 threads to the inch with nuts to fit obtained from Howard Clock Products, Inc., 260 Charles St., Waltham 54, Mass. Mica supplied in 1 in. (25 mm) squares by Eugene Munsell and Co., New York.

Directions for making the glue were given by Varian (16).

Precautions.—Many materials, in addition to their peripheral effects, cause severe side-reactions such as gasping, cyanosis, cessation of respiration or complete abolition of heart action for 20–60 sec. Such reactions invalidate the mesoappendix test, since they produce severe general vascular reactions which mask the purely peripheral effects of the substances injected. For this reason, such substances should be diluted until only peripheral vascular effects are obtained, with no evidence of the general side-reactions.

Comment by H. Stanley Bennett

Dr. Zweifach might have discussed various other illumination systems, such as the quartz rod of Knisely, Ultra-pac and Beck type of vertical illuminators. These devices allow observation of vessels which cannot be studied by the method he describes. A new development in this field is application of phase microscopy. The discussion of the care and methods necessary for preserving normal physiologic conditions at the observed area is excellent.

The following references contain additional techniques for microscopic study of living tissues:

Bennett, A. H.: Phase difference microscopy for transparent objects, Anat. Rec. 89: 547, 1944.

Bennett, A. H., *et al.*: Phase microscopy, Tr. Am. Microscop. Soc. 65: 99–131, 1946.

Ellinger, P., and Hirt, A.: Microscopic investigations of living organs: I. Method—intravital microscopy, Ztschr. f. Anat. u. Entwcklngsgesch. 90: 791–802, 1929.

Ellinger, P., and Hirt, A.: Method of study of living organs with highest magnification in fluorescent light (intravital microscopy), in Abderhalden, E. (ed.): *Handbuch der biologischen Arbeitsmethoden* (Vienna: Urban & Schwarzenberg), pt. 5, sect. 2/2, pp. 1753–1764, 1930.

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Knisely, M.: in Cowdry, E. V. (ed.): *Microscopic Technique in Biology and Medicine* (2d ed.; Baltimore: Williams & Wilkins Company, 1948).

Zernicke, F.: Phase contrast: New method for microscopic observation of transparent objects, Physica 9: 575–693; 974–986, 1942.

Comment by Harold D. Green

Dr. Kunze in our laboratory has experienced some difficulty with sodium pentobarbital. Urethane in a dose of 150 mg/100 g administered intramuscularly has proved to be more satisfactory.

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2. Chambers, R., and Zweifach, B. W.: Functional activity of blood capillary bed with special reference to visceral tissue, Ann. New York Acad. Sc. 46: 683, September, 1946.

cover, is made as is piece 1, except that the hole is 12–14 mm in diameter. The bottom, or piece 2, is a simple disk 1.5 mm thick and the full size of the lucite bar.

Other modifications (6) of the chamber have been described, such as the "bay" and "moat" chambers, developed for investigation of special problems in pathology and pharmacology (1). I have not used these types, but appreciate their value.

b) Stock for restraining animals.—A satisfactory restraint should meet the following requirements: (1) the animal must be able to assume a natural position; (2) it must be comfortable; (3) movements must be strictly limited. A stock is made in which the animal can stand in a normal manner. The belly is supported by a wooden or canvas crosspiece, and canvas is drawn over the animal's back and laced securely. A yoke is placed over the neck and held in place by winged screws. The head is kept in a constant position by a nose-piece that reduces lateral movement to a minimum. An animal in such a stock may be observed for long periods without great difficulty. The ear can be placed under the microscope without distortion or twisting.

PROCEDURE

For best results, strictly sterile precautions must be observed throughout the operation.

All plastic chambers used to date have been injured by boiling, so they must be sterilized by chemical means. The chambers are immersed in a 1:1000 aqueous solution of merthiolate at least 2 hr before being used, then washed in sterile saline solution and placed on the sterile table for the operation.

The ear of the dog or rabbit is clipped or shaved, washed with carbon tetrachloride compound (Ohio Chemical Manufacturing Co.), painted with iodine and draped. The operation as practiced in the Division of Experimental Medicine of the Mayo Foundation is done in the following steps, *after the animal is anesthetized with pentobarbital sodium.*

1. A large cork 6–8 cm in diameter is placed under the operative field. Piece 2 of the chamber is placed on the ear so that the central table over which the vessels are to grow lies close to the medial artery. This position is necessary for proper ingrowth of vessels.

2. Straight needles (no. 8 embroidery) are inserted through the three holes of piece 2, thrust through the ear tissues and into the cork beneath. Piece 2 is then removed. The needles orient the operator as to position of the threaded rods and the area over which the skin must be freed.

3. With a sharp scalpel a small area of the skin is separated from the underlying tissues. An effort is made to find a line of cleavage that will preserve as many subcutaneous blood vessels as possible.

4. A blunt instrument is used to free the skin over the entire area to be occupied by the chamber and for a few millimeters beyond.

5. Steps 3 and 4 are repeated on the lower surface of the ear.

mm thick. Drilling is not prolonged enough to generate sufficient heat to cause sticking.

2. Before the disk is cut from the bar, a depression about 15 mm in diameter is made in the center of the bar with a beveling tool, leaving the thickness in the center about 0.5 mm.

3. After beveling, a disk 1.5 mm thick is cut off with a narrow cutting tool. This disk will become the upper or covering portion of the chamber, hereafter called piece 1. Beveling is done to aid in manipulating the high power objective of the microscope over the chamber.

4. To make the lower disk or bottom portion of the chamber (piece 2), the lucite bar is cut with a narrow cutting tool to a diameter of 7 mm. This forms the table on which the blood vessels will grow and is cut so that its surface is about 2 mm above the rest of the disk. The disk itself is cut from the lucite bar so that it will be about 1.5 mm thick.

5. Pieces 1 and 2 are placed in the position in which they are to be used and three holes 1 mm in diameter and 2 mm from the periphery of the disks are drilled so that they form an equilateral triangle.

6. After pieces 1 and 2 have been bolted together the edges may be ground off to form a triangular shape if preferred. A notch is cut at the same point in each piece to permit their orientation on insertion.

7. A thin layer of copal glue is spread over the lower surface of piece 1 and firmly pressed on a sheet of mica. Any excess of glue will be forced onto the portion of mica that will cover the transparent portion of the chamber and make it cloudy; this is to be avoided. The mica-covered disk is placed in a screw clamp until the glue has dried thoroughly.

8. Both surfaces of piece 2 are buffed with fine emery cloth, then polished with silver polish and a silk cloth. Polishing is continued until the central area is highly transparent and free of scratches.

9. To create a space into which the blood vessels may grow, the two pieces are separated by placing three buffers or separators on the table, or raised portion, of piece 2. The buffers are cut out 1 mm square from a *piece of kodaloid* 70 μ *thick. The three pieces are placed near the periph*ery of the table of piece 2 in the shape of a triangle. Ethyl acetate, a lucite solvent, is run under the kodaloid by touching the capillary portion of the pipet to the edge of the kodaloid; at once the solvent will flow under, dissolving the lucite and fusing it with the kodaloid. An excess of ethyl acetate must be avoided because it will *flow from under the buffers* and fog the chamber.

10. After the glue holding the mica to piece 1 has dried, holes are drilled through the mica to admit the threaded rods. The mica is trimmed about 2 mm from the lucite, then trimmed as close to the lucite as possible. Trimming in two stages assists in preventing cracking or loosening of the mica from the lucite. The edge is smoothed with a file or emery cloth.

The so-called preformed chamber, or the one used to protect the ear tissues from which the skin has been removed, is made so that a maximum of space is available for visualizing the tissues. The top piece, or

effort is required to restrain the dog and to get it into position for observation.

Critique.—By means of the transparent chamber, important contributions to knowledge of the anatomy and physiology of capillaries and other minute blood vessels have been made (5, 7, 13, 14). These chambers are well suited to investigations of the effects of drugs (1, 11), anesthetic (15) and pressor agents (2) and other material. Anaphylactic response (3), effect of traumatic shock (10), the problem of transient leukopenia (8) and the effect of frostbite (12) have been observed.

The behavior of fully developed blood vessels that have grown into a transparent chamber closely approximates that of peripheral blood vessels elsewhere in the body. The preformed type of chamber is recommended by Clark if the question of normality of the blood vessels and surrounding tissues predominates. The preformed type is much thicker, and consequently observations on minute structures such as endothelial cells are difficult if not impossible. However, if behavior of a large vascular area or of a large blood vessel is being investigated the preformed chamber is preferable. It proved admirably suited to investigation of injury and repair of peripheral nerves (9).

In studies of vasomotor responses considerable difficulty is experienced in detecting vasodilatation by direct observation. Vasoconstriction is fairly easy to detect. For recording changes the motion picture camera is ideal, but effective records can be made with the still camera. The vessels may be measured by a calibrated ocular micrometer, or a series of camera lucida drawings may be made to record changes induced by whatever means.

For classroom demonstration of the peripheral circulation the transparent chamber is unexcelled. This technique could be used to advantage by every laboratory of physiology in presenting to students the subject of capillaries and other minute blood vessels and the behavior of blood cells. The extent to which such a preparation can be utilized in teaching is limited only by the imagination and ingenuity of the teacher.

Comment by Richard G. Abell

Since transparent chambers are composed of material foreign to the tissues, the question arises whether the vessels in such chambers are normal. It may, I believe, be answered in the affirmative, with one exception, discussed below. Let us see whether by morphologic and functional criteria the arteries, veins and capillaries in transparent chambers resemble corresponding vessels in the body.

Structure of the capillaries in the chambers shows no appreciable differences from that of capillaries in such naturally transparent tissues as the mesentery and omentum and in the tadpole's tail. Also, careful studies of capillaries in chambers have disclosed all of the important structures visible in fixed and stained sections. Thus morphologically the capillaries in chambers appear normal. This is equally true for the veins and arteries, but for one exception, namely, that not all arteries that grow in the chambers become supplied with nerves.

6. From the center of the area of skin separated from its subcutaneous tissue an incision is made radially to each needle. Care is taken that the skin is free of attachment to the subcutaneous tissues *throughout* the area into which the chamber is to be placed.

7. Into the lumen of a hypodermic needle, each of the orienting needles is placed in turn. The hypodermic needle is thrust through the ear tissues. Piece 2 of the chamber is held in correct position and a threaded rod with the lower burr in place is run through the proper hole in piece 2. The orienting needle is withdrawn and the bolt or threaded rod put into the lumen of the hypodermic needle, which is then withdrawn, and at the same time the threaded rod is carried through the tissues without difficulty. This is done in turn with each of the threaded rods.

8. The elevated portion of piece 2 is then visualized by transmitted light. With a sharp scalpel the cartilage of the ear is cut through about the periphery of the elevated central portion of piece 2. After excision of the disk of cartilage the upper half of the chamber is put in place, the upper burrs are placed on the threaded rods and the two portions of the chamber are brought closer together. The flaps of skin are brought out from between the two parts of the chamber and drawn over the periphery of pieces 1 and 2, and are then trimmed to the desired degree. The two halves of the chamber are brought closer together by further tightening of the burrs. When pieces 1 and 2 have been drawn together to the point where the three buffers are plainly visible the operation has been completed. A gauze dressing is placed around the ear with or without sulfanilamide powder dusted on the exposed cut surfaces.

The same procedure is followed when the preformed type of chamber is to be inserted except that the cartilage is not incised (paragraph 8). Elaborate dressings and protective coverings are not used by us with either chamber. The ingrowth of vessels may be seen as early as the fifth day after operation; in the best preparations, ingrowth has been completed in 30 days.

Some chambers may be of service for several months or even a year, but most of our preparations give a more limited period of usefulness. Early failure is frequently traceable to infection, but it must be appreciated that a foreign body is being placed in the tissues and a reaction may be expected.

Although we have placed the chambers in the ears of a considerable number of dogs, this species is far less satisfactory than the rabbit in several particulars. (1) The skin does not heal readily around the edges of the chamber, but continues to grow over the chamber, and for weeks there is constant oozing from the skin edges sufficient to fog the lens of the objective. (2) The vessels do not usually develop over the whole chamber as in the rabbit. (3) The dog is able to move its ears to a much greater extent than the rabbit, making observations on the chambers more difficult because the dog can pull the ear out of the field. (4) More

blood pressure, indicating that the arterioles in the chambers behave as do arterioles elsewhere in the body. On the basis of these various studies it is to be concluded that arteries in the chambers which are supplied with nerves behave normally and are not adversely affected by the materials of which the chambers are composed.

In experimental studies, the presence of arteries lacking nerves is of no particular disadvantage, for those supplied with nerves are readily distinguished and selected for observation. In fact, the presence of some arteries which lack a nerve supply is desirable for purposes of comparison.

It should be noted that my experience is limited to chambers made of kodaloid and mica, and glass and mica, and these comments apply specifically to chambers composed of these substances.

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This was shown clearly by the Clarks and Williams, who stained nerves in a transparent chamber with methylene blue. Some arteries lacked a nerve supply, others were supplied with nerves through part of their length and others throughout their entire length. Arteries supplied with nerves were seen to undergo rhythmic, spontaneous constrictions which were not apparent in arteries not supplied with nerves. Also, nerve-supplied arteries can be distinguished from those lacking nerves by poking the animal, which causes marked constriction of the former in a few seconds, whereas the diameter of those not supplied with nerves remains relatively constant.

Numerous studies show that vessels in the chambers function in exactly the manner as do vessels elsewhere, except, of course, the arteries not supplied with nerves.

Concerning capillary function, we are particularly concerned with permeability. I have shown that when a substance known to diffuse rapidly through capillary endothelium, such as urea, is injected intravenously into rabbit ears containing moat chambers it passes quickly through the capillary walls. In contrast, the dye T-1824 injected intravenously passes very slowly through the capillary walls owing to its attachment to plasma proteins. With reference to these substances, then, capillaries in the chambers behave as do capillaries elsewhere in the body.

Another criterion of capillary permeability is the plasma-corpuscle ratio. Hematocrit studies show this ratio in blood to be 55:45. The actual ratio in capillaries in the chambers varies continuously according to the number of corpuscles floating through a given capillary at a given time, but the average ratio is at least as great as, and probably greater than, that of venous blood. On irritation of blood capillaries in the chambers or elsewhere in the body the ratio changes rapidly because fluid passes through the capillary walls, leaving corpuscles concentrated in the vessels. Such an increase in permeability is readily produced in capillaries in the chambers by pressing on the top of the chamber, applying a chemical substance such as alcohol or increasing the temperature sufficiently. Except on irritation in some such manner, the plasma-corpuscle ratio of capillaries in chambers is in the normal range. Furthermore, it is the same as that seen in the capillaries of mesentery and omentum under the high power microscope.

A characteristic of normal vascular endothelium is its ability to undergo striking changes in behavior toward leukocytes in response to various stimuli. In the absence of such stimuli the condition of the endothelium is such that leukocytes do not adhere to it, but roll along the walls of capillaries and venules without changing shape. When these stimuli become active the leukocytes adhere to the vessel walls in increasing numbers and finally migrate to the surrounding tissue. In this respect vascular endothelium in the chambers behaves exactly as it does elsewhere.

The rhythmic, spontaneous constrictions seen in arteries supplied by nerves are also observed in preformed tissue chambers, in which the original arteries of the ear, with their nerve supply, are present. With Dr. Robert Chambers I observed the same type of spontaneous constrictions of arterioles, metarterioles and precapillary sphincters in the transparent chambers as had been described by Chambers and Zweifach in vessels in dog omentum and rat mesoappendix.

Arteries in transparent chambers constrict markedly in response to pressor drugs, the duration of constriction corresponding closely to that of increased

perature is $T_1°$ and $V_{a \to b}\, T_2$ at the other junction whose temperature is $T_2°$; and these are oppositely directed in the circuit. The resultant Emf in the circuit, which will cause current to flow, is then $V_{a \to b}\, T_1 - V_{a \to b}\, T_2$.

In the case of some available metals, the variation of intrinsic potential differences at the junctions with temperature is such that, over the range from 0 to 40 C, this difference $V_{a \to b}\, T_1 - V_{a \to b}\, T_2$ is proportional to the difference of temperature $(T_1 - T_2)$. Without careful investigation it should not be concluded that this is true over a wider range.

Measurement of Emf in the circuit will permit measurement of the difference of temperature between the two thermojunctions, and if then the temperature of one junction is known (because the junction is

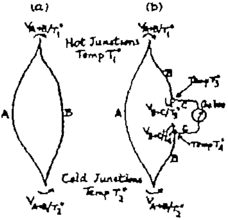

FIG. 1.—Principle of the thermocouple.

attached to the bulb of a thermometer in a Dewar flask), the temperature of the other can be calculated by simple arithmetic. Thus we have a "hot junction," held against the skin, and a "cold junction," the temperature of which is known. The combination is called a "thermocouple." Even if the strict proportionality between the Emf and the temperature *difference* between the junctions does not apply, the apparatus is usable by calibration when the temperature of the hot junction is also known, but in this case the temperature of the cold junction must always be the standard temperature at which the calibration was made.

Physicists usually measure Emf by a potentiometer, but for purposes of physiologic or medical research this is unnecessary. Instead, the current is allowed to flow through a galvanometer, and its deflection indicates the magnitude of the Emf that generated the current. If calibration is made directly, using the same galvanometer and current arrangement, there is no objection, even theoretical.

Introduction of the galvanometer into the circuit (Fig. 1, *b*) adds at least two more junctions where potential differences may arise, i.e., between the ends of the wire into which it was inserted, and the binding

III. Temperature of Skin: Measurement and Use as Index of Peripheral Blood Flow

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The good diagnostician makes constant use of his observations of the temperature of the skin of his patients by employing the temperature receptors of his own hands. (In this connection, it is a pity that many medical students are not taught that the backs of the fingers are more richly provided with receptors than the palmar surfaces.) Medical research and clinical investigation concerned with the peripheral circulation depend greatly on measurements of skin temperature, particularly the changes that occur when vasomotor nerves are blocked or surgically interrupted. *Measurement of skin temperature is one of the most useful* tools in medical research, but it is safe only in the hands of those who appreciate the underlying physical principles. It should be used in conjunction with other methods of research on peripheral blood flow. Clinical application of measurements of skin temperature is found in many texts, such as that of Abramson (1). Sheard's extensive discussion (25) has been freely drawn on for this review.

I. *Measurement of skin temperature.*—It is said that Humphry Davy in 1814 made the earliest measurements of skin temperature by holding the bulb of a thermometer against the skin. There are obvious errors, since the temperature reached by the bulb is affected by the temperature of the air; however, lacking more elaborate apparatus, valuable measurements can be made with a clinical thermometer. Stewart (27) showed that if the bulb were held in a cleft cut in a small cork and the exposed portion held against the skin, the rest of the bulb was insulated effectively and readings would deviate less than 0.5 (C) from those obtained by thermocouples or resistance thermometers. This accuracy is sufficient for most clinical investigation. Time must, of course, be left to insure that maximal temperature is attained.

More elaborate methods are all electrical and may be divided into the three categories that follow.

1. *Thermocouples.*—The most widely used apparatus is the thermocouple. The fundamental principle involved is that at every junction of dissimilar metals, as when a copper and a steel wire are twisted together, there exists a potential difference, due to difference in the state of the electrons in the two metals. The magnitude (intrinsic difference of potential) depends on the temperature of the junction, and this enables us to measure temperature by measuring the potential difference.

However, to measure the potential difference a circuit must be completed, which inevitably introduces at least another junction of dissimilar metals, the seat of a second potential difference (Fig. 1, *a*). There are thus two Emf's in the circuit, $V_{a \to b}$ T_1 at one junction whose tem-

able. The cost, exclusive of the galvanometer, need not exceed \$15; a suitable galvanometer costs \$50 to \$75.

Metals most suitable for the thermocouples are copper and various alloys, of which constantan (Advance) is the best known. This combination gives an Emf of about 40 μv for each degree (C) difference of temperature between the junctions and a closely linear calibration over the working range. Other combinations will give a greater Emf, but this is offset by greater resistance in the leads; where copper is not one of the two metals, great care must be taken to eliminate the effect of additional junctions in the circuit. The combination of steel and constantan is useful where thermocouple needles for taking temperature beneath the skin are required. The constantan wire is threaded through the steel barrel of an hypodermic needle and soldered to it at the tip, which is ground off to a sharp point. Steel lead wires must complete the circuit from the needle barrel. Steel with constantan gives about 50 μv per degree. The Emf obtainable from any combination is to be found from tables of thermoelectric power (e.g., *Handbook of Physics and Chemistry* [Cleveland: Chemical Rubber Publishing Company]).

The decision as to size of wire is a compromise between theoretical and practical considerations. Theoretically, the wires of the hot junction should be as fine as possible to minimize the cooling effect of conduction of heat in the metals, at the point of measurement on the skin. After fine wires have been repaired several times most investigators compromise on gauges of no. 28 (0.013 in. diameter) to no. 36 (0.005 in. diameter; Brown and Sharpe).

The *hot junctions* are made by twisting together the bared wires and soldering at the junction. Acid zinc flux gives the best results if it is at once washed away completely by 5 min immersion in running water. So-called noncorrosive fluxes, used by the radio industry, are unsatisfactory and inevitably give chemical Emf's unless washed off with solvents. Spot welding is highly satisfactory. The small ball of solder at the junction should be flattened with a hammer on a hard surface, when still soft, to form a thin disk a few millimeters in diameter. This is held in contact with the skin by strips of adhesive tape just behind the actual junction but not covering it. Where spot readings at several points are to be taken in succession with a single thermocouple, the junction may be supported between violin bridges mounted at the end of an insulated applicator, or other devices may be used to support the junction and press it firmly on the skin. It is definitely not permissible to have any insulated backing to the junction where it touches the skin, especially not the high thermal insulation found in some apparatus sold to measure skin temperatures. Such insulation interferes with normal heat loss of the skin at the point being measured, and the skin temperature will rise slowly during the measurement (8).

The *lead wires* should be separated and be insulated immediately behind the actual junction. They may individually be pulled through small

posts or lead wires of the galvanometer. These potential differences are denoted by $V_{b \rightarrow c}\ T_3$ and $V_{b \rightarrow c}\ T_4$, where C is the new metal of the galvanometer leads. If we insure that the temperatures T_3 and T_4 of these additional junctions are the same, the two additional Emf's will cancel each other. These additional, and unwanted, Emf's in any thermocouple circuit will exist and give trouble unless the following precautions are taken. (*a*) All additional junctions, as between leads and galvanometer binding posts, or at any switches that may be used, must be kept at the same temperature. For very accurate work they may be placed in baths of melting ice, but this is not necessary for the degree of accuracy demanded in measurement of skin temperature. It is sufficient to anchor additional junctions to heavy metal posts or heavy brass strips, placed close to each other and remote or insulated from sources of heat. (*b*) The

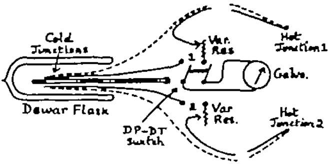

FIG. 2.—Circuit for double thermocouple. Solid line, copper; dashed line, constantan.

galvanometer, or switches, should be placed in that lead which is of the metal most similar to the copper of such instruments or switches, i.e., if copper be one of the metals of the thermocouple, in the copper lead rather than in the other. Any differences of temperature at additional junctions will then produce a minimal Emf.

Multiple thermocouples are usually required, so that temperatures of many points on the skin can be measured in rapid succession (Fig. 2). It is a mistake to suppose that an improvement can be made in this circuit by combining the various cold junctions into a common cold junction. Inevitably this leads to short circuits through the subject's body when the junctions are in place, and there will be chemical Emf's as well as other artifacts. The "fixed-variable" resistance is included for convenience, as explained later. It will be obvious how additional thermocouples could be included. Multiple contact double-gang switches of good quality, sold as radio parts, answer the requirements well.

PROCEDURE

a) *Apparatus.*—The thermocouple is easily constructed by following the instructions given here, and the result will usually be an instrument more adaptable to one's particular needs than any commercially avail-

able. The cost, exclusive of the galvanometer, need not exceed $15; a suitable galvanometer costs $50 to $75.

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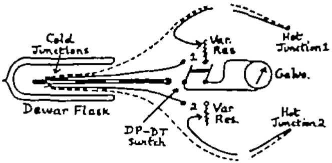

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PROCEDURE

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corrections. The deflections are then plotted against known differences of temperature, and the best straight line is drawn through the origin. Observations should be made for each set of multiple thermocouples, since differences may exist owing to differences in resistance of the various leads. From the slope of the line in the graph a calibration factor is calculated, i.e., the temperature difference in degrees that corresponds to 1 unit of deflection of the galvanometer. In use the deflection is observed, multiplied by this factor, and added to the temperature registered by the thermometer of the cold junction, to give skin temperature.

It is often convenient to alter resistance in the leads so that the factor has an integral value, such as 0.1° C for each division of the galvanometer scale. This may be achieved by inserting resistances (wound of manganin or other wire of low temperature coefficient and low thermoelectric power against copper) in the copper leads and adjusting these until desired sensitivity is achieved. If the leads are not sufficiently close to identical, a separate resistance will be needed in each thermocouple circuit. When the galvanometer is of high resistance compared to the leads, a single adjustable resistance in the common galvanometer leads may be used to bring all to the convenient calibration.

c) *Errors.*—For complete discussions reference should be made to books on physics, or see specific references given by Busse (16).

Parasitic chemical Emf's at the junctions are the commonest error. These can be detected by the fact that the calibration curve of deflection against temperature does not pass through the origin; i.e., there is a deflection even when both junctions are held at the same temperature. The only cure is the remaking of the junctions with more care to see that all traces of flux are washed off. Parasitic thermal Emf's from additional junctions of dissimilar metals in the circuits can be detected by warming all possible sites in turn with the fingers. When found, these junctions should be relocated so that their temperature will remain stable and opposing Emf's will accurately balance.

In work at low environmental temperatures, as in cold chambers, where the lead wires pass from the chamber to the warm outside laboratory, great care must be taken to eliminate parasitic thermal Emf's. The temperature of the chamber will affect resistance of the leads, so that the potentiometer method of measurement, which eliminates this effect, is preferable, or a high resistance galvanometer may be used to render the effect negligible.

In such cases it is safer to use resistance thermometers, in which these errors can be eliminated by use of compensating leads, described later.

2. *Radiation thermopile.*—To those who feel that application of a thermocouple to the skin seriously interferes with normal conditions and may change the skin temperature, the radiation thermopile is superior in that it offers a method of measurement without actual contact. Others think that the disadvantages offset this theoretical advantage. Disadvantages are that sensitivity is likely to be less, unless more sensitive and

bore rubber tubing and taped together. If both are placed in a single rubber tube, the insulation will soon be frayed in use and short circuits will develop. A convenient and cheap method is to mount the two wires between two strips of $^1/_2$ in. wide adhesive tape, with each wire about $^1/_8$ in. from the edge. The length of leads is adjusted to the particular problem.

The *cold junctions* are similarly twisted and soldered, then taped in close contact with the bulb of a thermometer readable to 0.1° C. The thermometer with its cold junctions is then mounted in a Dewar flask. If oil is used in the flask to maintain constant temperature, no insulation of the junctions is needed. However, oil is messy and has a low specific heat, and most workers prefer to insulate the junctions by pulling a thin rubber bag (obtainable from a druggist) over them and use water which, because of its high specific heat, changes temperature very slowly.

The *galvanometer* may be selected from a wide choice, since great speed of response is not required. Table galvanometers of the pointer type (Weston) or the enclosed portable mirror type (Rubicon; Leeds & Northrup) give sensitivities up to 1 mm scale division for 0.1° C, which is ample. Voltage sensitivity rather than current sensitivity is required. Thus a galvanometer of high current sensitivity, with many turns of fine wire on its coils and resistance of up to thousands of ohms, may give less voltage sensitivity than one of low current sensitivity but much less resistance. When current sensitivity is given (in terms of mm scale deflection/μamp), this should be multiplied by total resistance in the circuit (that of galvanometer plus lead wires) to give voltage sensitivity (in mm deflection/μv). A sensitivity of 1 mm deflection for 10 μv or less is sufficient for work with skin temperature. For maximal portability and ruggedness, with sensitivity, the type having a resistance comparable with that of the rest of the circuit (usually of the order of 50 ohms) is theoretically the best.

The time period of the galvanometer need not be less than 2 sec, greater time periods slowing its use. Care must be taken to see that the galvanometer is close to the condition of critical damping, i.e., approaches its final deflection without either oscillation or a very slow exponential drift. This is a matter of arranging the resistance in the external circuit to the proper value. If it is less than the critical damping resistance the instrument will be overdamped and slow; if too great, it will be oscillatory. Either by use of shunts across the galvanometer or by insertion of series resistances in the circuit, critical damping may be achieved, but, of course, only at the cost of sensitivity.

b) *Calibration.*—This is carried out by mounting temporarily the hot junctions in contact with the bulb of a second thermometer, just as the cold junctions are permanently mounted, and recording a series of deflections of the galvanometer with various differences of temperature between hot and cold junctions. The two thermometers should of course be compared with each other in the same beaker to determine necessary

during the measurements; the cold junctions must be arranged so that they will remain at room temperature. This feature is often lacking in available instruments, and there is resulting slow fall of deflection from the initial value reached.

The temperature deduced from thermopile measurements is the radiation temperature of the skin. It is possible that this is not exactly the temperature measured by thermocouples, though the difference is probably negligible. For instance, if the outer layer of the skin has any transparency to radiation, the thermopile may be effectively measuring temperature at a layer just beneath the surface.

3. *Resistance thermometers.*—Stewart (28) in 1891 described resistance thermometers, in which the sensitive elements were made from lead foil, for measurement of skin temperatures. The resistance thermometers then in use in the calorimeter of the Russell Sage Institute were described by Soderstrom (26). A type designed to give average skin temperature over large areas of the body was described by Burton (12).

The fundamental difference between resistance thermometer and thermocouple measurements lies in the fact that whereas with thermocouples the only energy available to operate the measuring instrument (galvanometer) is the thermoelectric Emf at the junction (and this is small), with the resistance thermometer the energy comes from an independent source (battery) and the temperature being measured merely controls this. Thus the method offers the possibility of using less sensitive and more rugged indicating instruments. It also lends itself to use of vacuum tube amplification; no one has found a way to amplify usefully, by vacuum tubes, the Emf from a thermocouple, the difficulty being the very low impedance of the input.

PROCEDURE

a) Apparatus.—The principle of the resistance thermometer is that certain metals, notably nickel and its alloys, have a high coefficient of change of resistance with temperature (up to 0.4 per cent/degree C). If a coil of such wire be held against the skin, skin temperature can be deduced from the electrical resistance of the coil at the moment. The coil is placed in 1 arm of a Wheatstone network; the other 3 arms are of wire of low temperature coefficient such as manganin (Fig. 4). Actual measurement of resistance would involve balancing of the bridge for each determination of skin temperature. The usual method is therefore to choose values of the balancing resistance (Fig. 4, R_1) such that the bridge is balanced for some particular standard temperature (say, 20 C). At any other temperature the bridge will be out of balance and a deflection of the galvanometer results. The amount of this deflection can be used to indicate the temperature of the coil. Thus the scale of the instrument may be directly marked in degrees C, and the complication of having to record also the temperature of a cold junction is avoided.

less portable galvanometers are used, and that temperatures beneath clothing cannot be taken. Thermopiles for measuring skin temperature have been designed by Aldrich (2), Bedford (7, 8) Hardy (20) and others. Construction of a thermopile is a matter for an instrument maker, whereas almost anyone can successfully construct a thermocouple. Only the principle is explained here.

A thermopile is a number of thermocouples connected in series, arranged so that alternate junctions (hot) are mounted behind a blackened disk which receives the radiation. The other alternate junctions (cold) are shielded from the radiation and remain at room temperature (Fig. 3). A cone of polished metal collects the radiation from an area of skin and reflects it on the receiving disk, the temperature of which rises until it is able to re-emit as much radiation as it is receiving. The excess tempera-

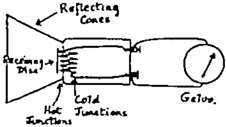

FIG. 3.—Radiation thermopile.

ture reached is indicated by the Emf generated in the thermopile and serves as a measure of the intensity of the radiation. By the law of Stéfan (based on thermodynamics as well as experimentally verified), the intensity of radiation emitted by a hot body (here the skin) is proportional to the fourth power of its absolute temperature. The nature of the emitting surface also affects the amount of radiation, denoted by introduction of a multiplying factor called "emissivity." The emissivity of human skin is very close to that of a "black body" (which is 100 per cent), and no instances in which skin is markedly different are known (21). The high water content of skin and the fact that water has a high emissivity for heat radiation make it unlikely that variations of emissivity could invalidate the use of a thermopile as a true indicator of temperature, but the possibility must be kept in mind. Since a fourth power law is involved, the reading of the indicating instrument cannot be linearly related to temperature, and calculations have to be made. This is a disadvantage over thermocouples, though a trivial one. The possible change of emissivity with extreme dryness of the skin has not been investigated.

The wires of the constituent junctions of the thermopile must be very fine and the receiver of radiation very light, or there will be appreciable thermal lag in reaching the final deflection. A point that should receive more attention is that the temperature of the cold junctions, usually mounted close behind the hot junctions, must not be allowed to rise

ment, possibly used in a vacuum tube voltmeter circuit, or in a simple bridge circuit with microammeter (10), is lacking.

b) *Errors.*—When the leads to the actual temperature-sensitive coils are long, errors will arise from change of resistance of the leads with room temperature since copper has a high temperature coefficient of resistance with temperature. If total resistance of the leads is very small compared with that of the coil these changes may be negligible, but there is a simple automatic means of eliminating them. This is by use of compensating leads, identical in structure and length to those going to the coils and running with them but soldered together at the far end (Fig. 5). These leads are inserted in series with the balancing resistor R_2, and any change of resistance in the leads due to room temperature will be compensated (in this case, of course, the bridge must have equal ratio coils R_3 and R_4, and

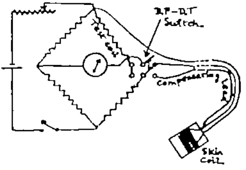

FIG. 5.—Use of compensating leads.

R_2 will be approximately equal to R_1). If desired, one of the four leads (the two to the coil and the two compensatory leads) can be eliminated, so that there are three wires only to each resistance thermometer.

It must never be forgotten that a resistance thermometer is itself a source of heat, from the current flowing in the coil, and therefore its temperature must be theoretically higher than its surroundings and the skin with which it is in contact. The current in the bridge must be kept low enough, and insulation between wire and skin made sufficiently small, so that the difference of temperature is negligible. A test of this is to immerse the coil in water and then in glycerin at the same temperature Since these liquids have thermal conductivities that lie above and below that of fully dilated and fully constricted skin, respectively, any significant error due to this cause will show itself.

This source of error in resistance thermometers can be turned to good advantage to measure directly the effective thermal conductivity of the skin, which varies with blood flow (14). The temperature of the coil, as indicated by deflection of the bridge when there is a small current in the bridge, is compared with the higher temperature registered when the bridge current is increased. The difference will be inversely proportional to the thermal conductivity. Instead of altering the current in the bridge, a second temperature-sensitive coil on the skin at a nearby point, ar-

When the bridge is used thus, the current from the battery must be held at a standard value. Some have used a second galvanometer or milliammeter to do this, adjustment to the standard value being made by the variable resistor in the battery circuit. This is not necessary, however, since by simply substituting, for the temperature-sensitive coil, one of fixed resistance (R_5), the bridge may be thrown out of balance by a fixed amount (corresponding to a fixed temperature of the "skin" coil). A test deflection results, and this is brought to the standard value by the battery resistor.

The resistance thermometer circuit is capable of many useful modifications for specific purposes. For example, both R_1 and R_2 may be made of temperature-sensitive wire, and R_1 placed on the skin, R_2 in the air or both on the skin at different points. Deflections of the galvanometer will

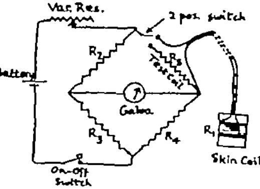

FIG. 4.—Circuit of resistance thermometer.

then give directly the difference of temperature between the two coils.

Resistance thermometers are easily constructed. Hytemco wire (Driver-Harris Company) has a high temperature coefficient and is obtainable in very small gauges with high resistance per unit length; no. 40 gauge (Brown and Sharpe) may be used without too many breakages. Coils for contact with the skin may be wound on frames of very thin bakelite fiber and lacquered with glyptal or other insulating varnish. They are held to the skin with adhesive tape as are thermocouples. When average temperature of large areas of the body is required, as in calorimetry, the wire can be held by sewing in a sewing machine against the inside of light gauze garments. This is a much more convenient way of obtaining average skin temperatures than computation from thermocouple readings taken at many points.

A new possibility of application of resistance thermometry to clinical measurements of skin temperature is offered by the discovery of Thermistors (6). These are used in radar research and were developed by the Germans for infra-red detection. They have high resistance in a very small compass and an extraordinarily high change of resistance with temperature. Wide experience with these for skin temperature measure-

II. *Interpretation of skin temperature in terms of peripheral circulation.*—1. "Normal" levels of skin temperature. The level of the temperature of the skin depends on two sets of variables, physical and physiologic, in each of which there are several factors. The physical factors are environmental temperature, amount and character of clothing worn and, to a less degree, humidity. The physiologic factors are mainly the location on the body and the blood flow to the skin at that point, although body build, amount of subcutaneous fat and blood flow of the rest of the limb and of muscles beneath the skin also play a part. In addition, evaporation of perspiration can so affect skin temperatures that their interpretation in terms of blood flow is very difficult.

It is not astonishing then that no set of "normal" skin temperatures can be defined, even when the physical factors are strictly prescribed. Even if biologic variability be excluded by choosing one individual, that individual's physiologic state is difficult to define and is widely variable within "normal" limits. For example, the skin temperature of the toes of a normal subject lying in a room at 20 C may be only just above room temperature or as high as 35 C, according to the state of general vasoconstriction or vasodilation, which depends on past history of his exercise, eating and even emotional state.

Comprehensive reviews of the level of skin temperature (e.g. (9) and (17)) are not much help because of the complexity and variability of the factors concerned. A rough guide to the levels to be expected was provided by Eddy and Taylor (18) from observations on 50 normal medical students. Thus absolute levels of skin temperature, even under controlled conditions, are not of great significance. It is the changes in skin temperature that occur during physiologic experiments or in pathologic states that are important.

2. Least change in skin temperature that is significant. With modern instruments, skin temperature could be measured to an accuracy of 0.01° C, but this would be purposeless in view of uncontrollable physiologic variations. Even in the best controlled physical conditions, fluctuations in skin temperature up to 0.5° C or more occur without ascertainable physiologic cause, and it is wise not to interpret as significant any changes less than this. Some guide as to significance is given from simultaneous measurements by Foged (19) of skin temperatures of bilaterally symmetrical areas of the body. He concluded that a difference of more than 1° C indicated abnormality. This agrees with some observations of our own on symmetrical areas of the back. In 50 pairs of observations the mean difference was only 0.02 C, with standard deviation of the differences ± 0.3 C. By statistical criteria this would mean that differences less than 0.9 C were not significant.

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ranged so that the current in it and thus the heat generated is insignificant, may be balanced against the first coil where more heat is generated. Deflection of the galvanometer then gives the difference of temperature (and hence conductivity) directly. The method offers many possibilities for thermal stromuhrs in various applications.

4. *Comparison of electrical methods.*—The advantages and disadvantages of the thermocouple, thermopile and resistance thermometer are

TABLE 1.—Comparative Evaluation of Electrical Methods

	Thermocouples	Thermopiles	Resistance Thermometers
1. Ease of construction	Easy	Difficult	Fairly easy
2. Cost of construction	Low	Higher	Low
3. Sensitivity	Limited by thermoelectric power of metals	Less	Not limited by thermoelectric power
4. Instrumental lag	Can be made very low	Slow unless vacuum thermopiles	Greater than thermocouples
5. Galvanometer required	Moderately sensitive, fairly portable	Quite sensitive	Relatively insensitive, rugged and portable
6. Possibility of electronic amplification	Not practical	Not easy	Easy
7. Errors likely	Parasitic Emf's sometimes troublesome; change in resistance of leads with temp.	Rise of cold junction temp.	Heat in coils due to current; leads can be automatically compensated
8. Calibration curve	Closely linear in working range	Not linear; calculation required	Closely linear
9. Ease of measurement	Cold junction temp. must be read	Room temp. must be read	No observations except direct scale reading
10. Breakage	Fairly common with fine junctions; thermometers break	Not common; thermometers break	Fairly common; no thermometers to break
11. Interference with skin temp.	Possible if not properly made	None, no contact	Possible if current too high
12. Range of application	Good	Not under clothing	Good
13. Size of area measured	Can be very small; hard to average over large areas	Area not less than 1 sq cm	Area not less than 0.5 sq cm; gives average of large areas
14. Other possible application	Differential thermocouples for special purposes	Not developed, but possible	Differential temp. measurement; thermal stromuhrs (thermistors)

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the room is then raised to, say, 28 C, the skin temperature will, after adaptation, rise markedly and remain high. Obviously vasodilation in response to demands of temperature regulation is one factor causing this rise. However, even in an inanimate model, in which thermal conductivity remained constant, there would be a rise of surface temperature on elevation of environmental temperature. The problem then is to determine how much of the observed rise is purely physical and how much is physiologic, due to an increase in peripheral blood flow.

Attempts have been made to define the rise of skin temperature to be expected for a given rise of room temperature, and even to provide formulae (8, 24), none of which has any wide field of application. In fact, from the theory of heat flow applied to the problem, the rise of temperature for each degree rise of room temperature, even in the inanimate model, would depend entirely on the level of skin temperature (11), varying from 0.8 C if the skin temperature be 23 C (in a room at 20 C) to 0.2 C if the skin temperature be 35 C. Again, the rise of skin temperature that would result from a given increase in peripheral circulation obviously depends on the level of skin temperature. If the skin temperature be low it will rise markedly, but if the skin temperature is already high (as on the trunk) it will rise much less for the same increase in blood flow. Even an infinite increase in circulation could not raise it above 37 C (rectal temperature).

4. Thermal circulation index. All these difficulties are resolved if a thermal circulation index, derived from skin temperature, be used (11). It is based on a physical analysis of the flow of heat from the interior of the body, where it is generated, to the surface. The index is such that if the circulation did not change, it would be a constant, even if the physical factor of room temperature had been altered.

Details of derivation of this index are given in the original paper (11). The temperature of the body surface must be lower than that of the interior (rectal or "deep body" temperature) in order that heat shall flow to the surface and so into the environment from the skin. There are a physiologic gradient of temperature, given by the difference between rectal and skin temperatures, and a "physical gradient" between skin temperature and air. The flow of heat down each of these gradients is governed by the law of diffusion of heat, i.e., for the physiologic gradient,

$$H = C'(T_r - T_s), \tag{1}$$

where H is the heat flow (cal/sq cm/sec), T_r and T_s are rectal and skin temperatures, respectively, and C' is a parameter expressing ease with which heat flows in the tissues. C' may be called the "effective thermal conductivity" of the tissues. It depends mainly on the blood flow in the tissues, though also on proportion of fat, water content of the tissues and, of course, length of the thermal pathway through the tissues to the particular point on the skin. In physiologic experiments, changes in C' will mainly be due to changes in blood flow. Determination of effective ther-

mal conductivity, C', is the object of research with skin temperatures, and its determination is the closest we can come, by observations of skin temperature, to a direct measurement of peripheral blood flow.

For the physical gradient, the similar equation is

$$H = C(T_s - T_a), \tag{2}$$

where the new symbol T_a is the environmental temperature, and C the effective thermal conductivity of the environment. If calorimetry is possible, so that heat flow H of equation (1) can be measured, C' can be directly calculated (15, 29). Calorimetry is not necessary, however, since we can combine the two equations, remembering that *in the steady state* the heat flow H of equation (1) arriving at the skin must equal that leaving the same area (H of equation (2)). Eliminating H by this identity we have

$$\frac{C'}{C} = \frac{T_s - T_a}{T_r - T_s} = r. \tag{3}$$

r is the thermal circulation index.

$$\text{Thermal circulation index} = \frac{\text{external physical drop of temp.}}{\text{internal physiologic drop of temp.}}.$$

The calculation is simple, but if desired a nomogram can be constructed from which the index may be calculated graphically (Fig. 6).

In physiologic experiments, the physical factor C, conductivity of the environment, can usually be kept constant. It will remain constant, despite changes in room temperature, if the degree of air movement and amount of clothing are not changed. It does not alter significantly with humidity. The changes in value of the index will then reflect changes in conductivity of the tissues; i.e., they will be indicative of changes in peripheral circulation.

a) *Dependence of index on location on body.* The value of conductivity C', the ease with which heat flows to a given point on the surface of the body, obviously depends on the total thermal pathway from a point where the temperature may be considered to be deep body temperature to the particular skin area. The last part of this pathway, in the skin of the area, may be the most important obstacle to flow of heat, but all of the pathway influences the value of the index. How greatly the rest of the pathway, other than the skin, may be concerned is shown by the fact that the temperature in peripheral arteries is not, as often assumed, close to the deep body temperature, but may be several degrees lower (5). Measurements made at different points of the skin indicate that the thermal circulation index may be 5–10 times as great for the toes as for the trunk, obviously because of the much larger thermal pathway. In practice then, the change in index that occurs at any given point on the skin is used as an indication of the change of blood flow, rather than the comparison of indices for different parts of the skin.

b) Dependence of index on blood flow. The effective thermal conductivity of the tissues depends on a combination of the true "conductivity" of the tissues, which would exist even if blood flow were zero, and the convec-

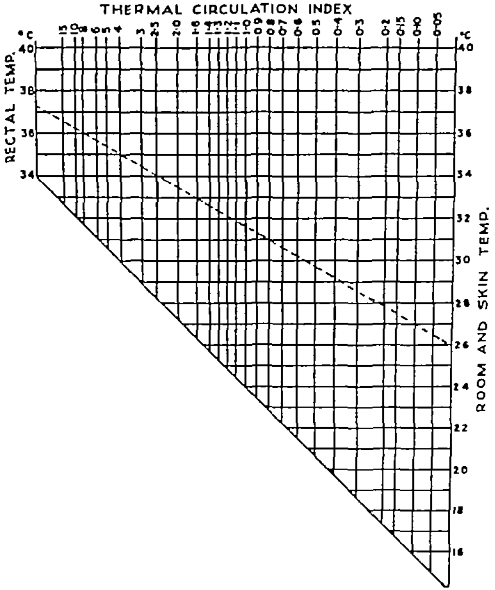

FIG. 6.—Nomogram for calculation of "thermal circulation index." A thread or ruler is laid across the chart to join rectal (left side) with room temperature (right side). The horizontal line corresponding to skin temperature is followed until it intersects the diagonal. The vertical line nearest the point of intersection gives the thermal circulation index. (Figs. 6 and 7 reproduced from A. C. Burton, in Moulton, F. R. (ed.): *Blood, Heart and Circulation*, Publ. no. 13, Am. Assoc. Adv. Sc., 1940.)

tive transfer of heat by the flow of blood. Various formulae that could be devised to give a plausible relation between effective conductivity and blood flow would have only theoretical interest. Comparison of thermal conductivity of skin and blood flow of the fingers, measured independ-

ently and directly (14), has shown a linear relation. However, if the thermal conductivity index rises by some factor, say 3 times, the blood flow to that point must have increased at least by this factor, i.e., must be at least three times as great. For the toes, the thermal circulation index may increase between full vasoconstriction and full dilatation (ether anesthesia (11)) by as much as 12 times. This increase represents the change in the whole thermal pathway to the toes, and a merely local dilatation in the toes will not cause as great a change in index, nor will the temperature rise as high.

III. *Critique*.—The following cautions in interpreting skin temperatures in terms of the peripheral circulation apply whether the thermal circulation index or skin temperatures alone is preferred.

1. Effect of blood flow in structures other than skin. Since skin temperature depends on the ease with which heat flows in the total thermal pathway to the point of measurement, the blood flow in muscle, which is a major part of the total circulation in a limb (4), may influence it to an unknown extent. Since blood flow in muscle in many circumstances may change in the direction opposite that in skin, the effects may be very complicated. Research on this point is overdue. An example is the rise of thermal conductance in water bath experiments (and therefore the rise in skin temperature in experiments in air), when the environment is cold enough to stimulate thermal muscular tone and shivering (15). Despite full vasoconstriction, which has come on even at higher temperatures, this increase in skin temperature occurs. It may well be that the true flow in skin has not increased, but is still minimal, and that the increase of blood flow in the active muscles, which influences part of the total thermal pathway, is solely responsible for the rise.

2. Nonsteady states; thermal lag. In the steady state, i.e., when skin temperatures are approximately constant, thermal conductivity of tissues is the variable on which they depend, but when temperatures are changing, the thermal capacity of the tissues also enters the equations of heat flow. Thus the thermal circulation index has no strict application unless the steady state is approximated, nor will momentary levels of skin temperature be related directly to the blood flow at that moment. For example, if blood flow falls abruptly, the skin temperature cannot fall immediately to the new level, associated with a steady state with the new low blood flow. Moreover the rate of fall depends on the total thermal capacity of the tissues involved; the fall is slower in a large limb than in a small one. A thermal lag is exhibited by skin temperature when this is changing.

Because of thermal lag the skin temperature fails to follow accurately the time relations of change of blood flow even when these are small and local (13). By the assumption of a lag of about 20 sec in this case, fair correspondence can be achieved, but there are slow trends in skin temperature which depend on what the blood flow has been doing in the previous several minutes. When the change of blood flow is toward a sus-

tained high level, as in general vasodilation, the temperature will rise rapidly, but still with a lag of many minutes in reaching the final level (13) (Fig. 7), but when blood flow is toward a low value the lag will be much greater (consistent with the theory of heat flow).

Most important is the consideration that when the change of blood flow is not sustained but brief, followed by return to the former levels, the changes in skin temperature are inadequate to indicate either the moment or magnitude of this brief change (13). Therefore, skin temperature measurements are a poor tool, compared with direct measurements of blood flow in limbs, in research on the effects of drugs or physiologic effects that are of brief action.

The rate of change of skin temperatures when the steady state has been disturbed can be put to use in another aspect of effective thermal

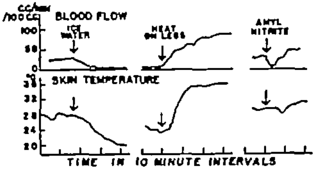

FIG. 7.—Simultaneous measurements of blood flow and skin temperature of a finger after various procedures. Large thermal lag of skin temperature is apparent, and inadequacy of skin temperature to indicate time and amount of transitory changes in the circulation.

conductivity of tissues. Unlike the thermal circulation index, measured in the steady state, the rate of change involves both thermal capacity and thermal conductivity, but the former may be often considered constant during the experiment. When the blood flow of a limb changes abruptly to a new sustained level, the curve of rise of skin temperature, except for the initial period of acceleration, will be exponential (23). A plot of the logarithm of the temperature rise that is still to go before the final level is reached, against the time, will then be linear (Fig. 8). The slope of this line is an index of effective thermal conductivity during the transition. The graphic method helps to verify that the curve is exponential, i.e., that the change in circulation was actually abrupt and sustained.

This method is limited by the fact that changes in blood flow are not always abrupt nor sustained. However, a useful adaptation is to observe the curve of return of skin temperature when it is caused to change locally by an applicator, colder or warmer than skin temperature, applied to the point on the skin (22). Here the time constant of the curve of change of skin temperature indicates the local effective thermal conductivity. In essence the method is analogous to that of observation of the blood flow of the skin by watching the rate of return of color after local

pressure occlusion. It offers a measure of local skin flow, whereas the ordinary use of skin temperatures gives information not exclusively concerned with blood flow of the skin. The limitation is that the local application must not produce any vascular change.

3. *Effect of evaporation of perspiration.* Equation (2) *is not strictly* correct, because loss of heat from an area of skin to the environment is partly by means of evaporation of perspiration, insensible and sensible, and rate of evaporation depends not only on skin temperature but on how wet the skin is, i.e., effective vapor pressure at the skin. Thus if, during

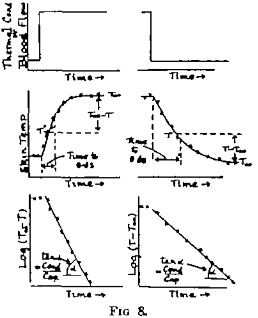

FIG 8.

experiment, the wetness of the skin increases because of sweating, heat loss of the skin increases and its temperature falls, even if blood flow has remained constant. Because of this, a fall of skin temperature during an experiment cannot be interpreted as indicating a decrease in peripheral blood flow unless sweating has been ruled out as a cause. However, any rise in skin temperature must be due to an increase of blood flow, although if there has been sweating the observed rise is less than it would have been otherwise. Lack of this caution in interpreting skin temperature led to the supposition that peripheral blood flow decreased in exercise, based on observations that skin temperature after heavy exercise was lower than before (9). Actually there is peripheral vasodilation in exercise, but the heavy sweating and evaporation in exercise can actually lower skin temperature despite this (12). Theoretically the thermal circulation index could be corrected for the effect of changes in evaporation, but only if the magnitude of these changes were known (11).

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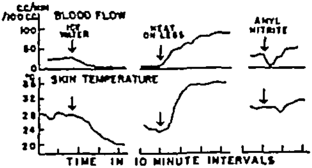

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Comment by H. C. Bazett

This is an excellent presentation of the practical handling of the relatively simple equipment needed. Occasional statements can be misinterpreted. Thus the author draws attention to the higher thermal sensitivity of the back of the fingers as compared with that of the palmar surfaces and accounts for the contrast on a basis of relative distributions of thermal receptors. The explanation may be valid but should not be regarded as fully established. Valid comparisons of distribution of receptors are hard to make since skins have very different textures and thicknesses. Since the histologic structures involved and their depth from the surface in these areas are not adequately determined, the uncertainties are great.

Dr. Burton points out the physical factors that may cause discrepancies in the normal parallelism that exists between surface temperatures and blood flows. He also refers to work on the cooling of arterial blood in transit as the result of heat exchange with cold blood returning in adjacent veins. However, he has not pointed out how complicated may be the conditions regulating physical transfer of heat, if this physiologic factor plays an important role. The existence of such complications is highly probable on the basis of simple theory, so that it can be accepted, even though it has not been adequately demonstrated.

The initial demonstration of exchange of heat between adjacent arteries and veins was made by Claude Bernard over 70 years ago. Recent advances merely have made evident that this exchange can be of considerable magnitude and importance. The rise in thermal conductance of the tissues that occurs during shivering is not explained here. This rise may depend partly on thermal exchanges between artery and vein, in which the arterial blood is warmed rather than cooled. The arterial supply of the skin arises from muscular branches and the arteries concerned pass through the muscles to reach the skin. The blood flowing through these arteries should be warmed by gain of heat from veins leaving an active muscle and would conduct, therefore, some of this heat directly from the muscle to the skin. The consequent improved thermal conductance would be advantageous in exercise but a liability in shivering reactions.

In individuals with vasoconstriction from exposure to cold, cooling of arterial blood in passage to the periphery is useful. Under warm conditions much of the venous return is obviously directed from a course in the venae comites to one in the superficial cutaneous veins. This diversion should decrease the precooling of arterial blood, and the decrease should be advantageous in warm conditions. Such variations should cause quantitative discrepancies to be found between changes in surface temperature and those in blood flow. Such discrepancies from linear relationships are recognized and may have many causes. One may be the physiologic adjustment under discussion. There are no adequate data to allow an accurate estimate of the modification of thermal conductance attainable by such adjustments.

Comment by Harold D. Green

For prolonged temperature registration we have found it highly satisfactory to attach the actual thermocouple to the skin with a drop of flexible collodion, attaching the leads at a short distance with adhesive tape. Less error appears to be caused by the thermal insulation provided by the thin layer of collodion than by an unnoticed air gap between the skin and the thermocouple.

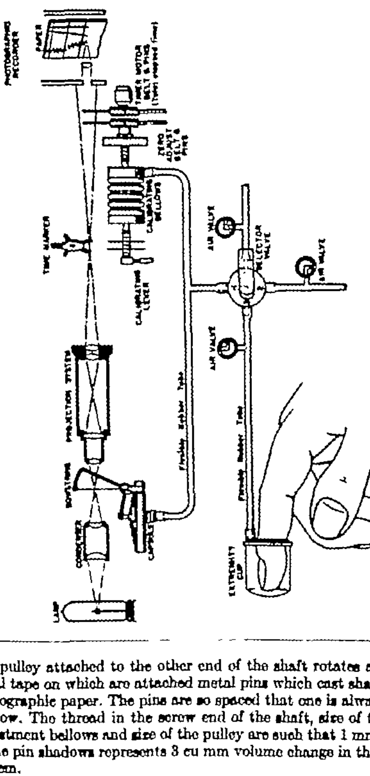

Fig. 1.—Diagram of pneumatic system and recording parts of plethysmograph.

The pulley attached to the other end of the shaft rotates and moves a metal tape on which are attached metal pins which cast shadows on the photographic paper. The pins are so spaced that one is always casting a shadow. The thread in the screw end of the shaft, size of the base-line adjustment bellows and size of the pulley are such that 1 mm movement of the pin shadows represents 3 cu mm volume change in the pneumatic system.

On the other end of the housing of the base-line adjustment bellows is a screw fixed to a calibration lever (Fig. 1). By means of stops adjusted

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IV. Sensitive Portable Plethysmograph

GEORGE E. BURCH, *Tulane University*

Plethysmography is not a new method of study in physiology, but its use for study of peripheral blood vessels has been limited to the laboratory because of the complicated nature of the apparatus and difficulties of maintenance. The plethysmograph described here has the advantages of being portable and about as simple to employ as the electrocardiograph (1). Its practical nature should permit its application in human and general physiology, psychiatry, normal and abnormal vascular states and general clinical and experimental medicine.

APPARATUS

The plethysmograph* consists of a sensitive volume-recording metal dome-shaped diaphragm or capsule which activates a mounted bow and bow string, lighting and lens systems for focusing the bow string, a coarse volume-recording system, timers, a camera and a selector valve (Fig. 1).

The pneumatic system consists of the sensitive metal diaphragm or capsule, a metal bellows to control the position of the bow string and an extremity cup, all connected at atmospheric pressure by thick-walled 1 mm rubber tubing (Fig. 1). With the pneumatic system closed, any change in volume of the part enclosed in the extremity cup causes a change in volume of the capsule, movement of which activates a short arm of light aluminum tube shaped essentially like a question mark around a pin set in jewel bearings. The long bow-shaped end moves 40 times the distance of the short arm. It carries a fine quartz bow string which is magnified about 150 times and focused by lenses on moving photographic paper of an electrocardiographic camera. Photographing can be done in a room with ordinary lighting. The selector valve is connected to three rubber tubes which may be connected to three extremity cups. (This can be modified to include any desired number.) By means of the selector valve any of the three parts can be selected for successive study. The coarse recorder bellows or base-line adjustment is also connected to the pneumatic system. Any change of volume of this bellows shifts the position of the capsule, thus moving the bow and bow string.

* Manufactured by Cambridge Instrument Company, New York.

The recorder should be adequately damped to record volume changes accurately. Overshooting and overdamping result in distortions in recorded volume changes. A mechanical device to produce a constant change in volume at desired rates from very low to very high frequencies by compressing and releasing a metal bellows is connected to the pneumatic system.

PROCEDURE

The conditions and method for recording the plethysmogram described here are intended for relatively ideal conditions, although good work can be performed under less satisfactory conditions.

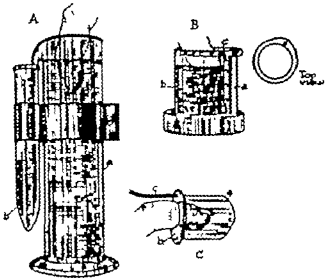

FIG. 2.—Apparatus for measuring volume of part for study. In *A*, water displaced by part spills from cylinder *a* to graduated test tube *b* for measurement. In *B*, water displaced by part displaces a meniscus in narrow bore tube *a* which connects with cylinder *b* near base; *c*, millimeter scale. *C*, extremity cup in place

a) *Physical environment.*—The type of observation room is of greatest importance (9). Temperature of 75 F and relative humidity of 50–60 per cent, with no drafts, are good conditions for a resting subject. The room should be pleasant and simply decorated to resemble one in a home. This leads to relaxation and reduces anxiety, apprehension and other disturbed psychic phenomena. The subject should remove all clothing or everything except loose underwear. Covering with a sheet or blanket is permissible. The subject must not become cool or warm; he must be comfortable at all times.

The part for study should rest at the level of the heart. When the subject is supine and the legs are relaxed and slightly rotated externally, the tips of the toes are at about heart level and need no support. For the finger-tips an adjustable arm rest is preferable, though pillows may be

by set screws, partial rotation of the calibration lever effects a 10 cu mm change in volume in the pneumatic system. This change results in movement of the bow string, making it possible to convert linear change in the bow string shadow of the completed plethysmograph to cubic millimeters of change in volume of the part enclosed in the extremity cup.

Since the parts of the extremity (finger- and toe-tips) vary in size, sensitivity of the bow string must be adjusted to reduce all linear movements of the string shadow to a standard unit of volume of a standard size part (5 cc is used since average size of the tip of the index finger is 5 cc). Sensitivity of the bow string can be varied to permit selection of a relatively large or small amount of movement of the string shadow for a given change in volume in the pneumatic system (*vide infra*).

The time that elapses when the camera is off is recorded automatically by a master timer or elapsed time indicator which rotates a pulley and metal tape. Fixed to the tape are four pins of different diameters which cast shadows of different widths on the plethysmogram. The rate of rotation of the timer shaft and size of the pulley are such that 4 mm of movement of a timer pin shadow is equivalent to the elapse of 1 min. From the width of the timer pin shadows and their relative positions before the recording is stopped and those at the beginning of the next interval of recording, the time the camera was off is measurable to a fraction of a minute (see Fig. 3). This is of value in study of the effects of stimuli, drugs and other procedures.

Horizontal millimeter lines and time marks are like those used in electrocardiography. Simple rotation of a lever changes the speed of the camera from 9.5 cm/min (slow) to about 145 cm/min (fast). Light intensity, timers and camera speed are changed with each simple rotation of the speed control lever to assure good recording at the two speeds.

The extremity cup (Fig. 2, *C*) is a thin-walled plastic test tube with one end closed by a diaphragm of the same plastic. The diaphragm has a hole shaped to fit the finger- or toe-tip loosely. Entering the lumen of the cup through the diaphragm is a thin-walled brass tube to which is connected the rubber tubing from the plethysmograph. A fairly large assortment of cups should be available to allow a fit for any size of finger or toe. The cups are chosen to insure minimal dead space without the part's resting against the wall.

The crucial portion of the instrument from the physical point of view is the sensitive metal capsule or diaphragm, made of 0.002 in. aluminum sheeting. The natural frequency of the recording membrane should be four or five times the most rapid frequency of the vibrations to be measured. Ordinarily, maximal frequency of the most rapid rates of volume change is found in the upstroke and dicrotic notch of the wave of the volume pulse. These have a frequency of about 10 c, averaging 6–7 half-cycles or deflections. The natural frequency of the metal capsule is about 150 c. This more than meets the requirements and makes the instrument suitable for study of animals with very rapid pulse rates, such as the rat.

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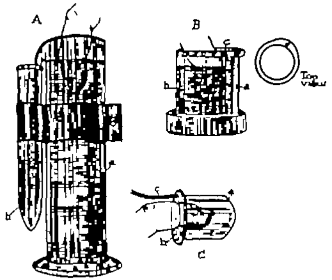

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used. It is imperative that the extremity cup touch nothing. The arm must be free from any torsion of the blood vessels.

No special preparation of the subject is required, although it is best that he refrain from eating, drinking, smoking and use of drugs long enough to avoid any influence on the blood vessels. He should be relaxed mentally and physically. The manner in which he is handled and the conduct of the observer during the recording are extremely important. A satisfactory recording can be obtained on the first attempt if sufficient attention is given the patient's psyche.

b) Measure of volume of part for study.—Highly accurate measurements to within several cubic millimeters (2, 3) are not necessary for usual clinical purposes. Two simple methods are used in this laboratory. (1) A lucite cylinder (Fig. 2, *B*, *b*) has a narrow vertical glass tube, *a*, fixed along its outer wall and connected with its lumen near the base. A millimeter scale, *c*, is placed near the tube. The internal diameter of *b* is such that an increase of 1 ml in volume of its liquid contents causes the meniscus in tube *a* to rise 0.5 mm. The cylinder is filled about three-quarters full with water. A detergent is placed over the fluid in tube *a* to prevent the meniscus from sticking. To measure volume, the part to be enclosed in the extremity cup is submerged in the water in the cylinder. The linear rise of the meniscus in tube *a* in millimeters produced by displacement of water in the cylinder indicates the volume of the part. Each 0.5 mm rise equals 1 cc of part. This method is accurate to 0.2 cc. (2) A large vessel (Fig. 2, *A*, *a*) on a horizontal platform is filled with water until it overflows into the calibrated (Esmarch) tube, *b*. The tube is removed from the metal clip, emptied and replaced. The part to be studied is gently inserted in the water until the portion to be included in the extremity cup is immersed. The water displaced spills into tube *b* and is equal to the volume of the portion of the extremity to be studied. The method is accurate to about 0.1 cc.

c) Connection of part to plethysmograph.—An extremity cup is selected that fits snugly without constriction. The diaphragm through which the part is inserted is shaped with a sharp knife. Sealing material (printer's roller compound brought to proper consistency with LePage's glue) is heated in hot water until it is viscous enough to be applied to make a tight seal. The same amount of part is enclosed in the cup that was immersed in the volume-measuring cylinder. After the sealing jelly has stiffened, the rubber tubing leading from the plethysmograph is connected to the metal tube on the cup diaphragm, fixed to the dorsum of the hand or foot and then forearm or leg with adhesive tape. This is important to prevent extraneous volume changes in the plethysmogram. When the extremity cups are being connected to the parts in the "open" and "closed" valves should be turned to "open." This connects the pneumatic system to the atmosphere and protects the sensitive metal capsule while connecting the part to the instrument. This precaution is exceedingly important. Simple turning of the valve to "closed" position isolates

the pneumatic system and part from the atmosphere at atmospheric pressure. The part is now ready for testing.

d) Focusing and adjusting of string shadow.—The string should rest at the center of the camera slit when the string is at the midposition of sensitivity with the pneumatic system open to the atmosphere. When the system is closed the string shadow can be brought to any position with the base-line adjustment. When the pneumatic system is open the string suddenly returns to original position. After the part is connected for study, the selector control is properly set and the valve for that part closed. The string shadow is then brought to the desired position with the base-line adjustment. If this is impossible there is a leak in the system, usually at the jelly seal at the extremity cup. The string shadow is then focused. By pressing on the calibrator lever until it is stopped, a 10 cu

TABLE 1.—Necessary Linear Movement of String Shadow for Various Sizes of Parts to Permit Standardization on Plethysmogram

Volume of Part, Cc	Mm String Shadow Must Move	Volume of Part, Cc	Mm String Shadow Must Move
1.0	50.0	5.2	9.6
1.2	41.7	5.4	9.3
1.4	35.7	5.6	8.9
1.6	31.3	5.8	8.6
1.8	27.8	6.0	8.3
2.0	25.0	6.2	8.1
2.2	22.7	6.4	7.8
2.4	20.8	6.6	7.6
2.6	19.2	6.8	7.4
2.8	17.9	7.0	7.1
3.0	16.7	7.2	6.9
3.2	15.6	7.4	6.8
3.4	14.7	7.6	6.6
3.6	13.9	7.8	6.4
3.8	13.2	8.0	6.3
4.0	12.5	8.2	6.1
4.2	11.9	8.4	6.0
4.4	11.4	8.6	5.8
4.6	10.9	8.8	5.7
4.8	10.4	9.0	5.6
5.0	10.0		

mm change in volume is produced. From the already measured volume of the part the string shadow is adjusted in sensitivity until a 10 mm movement of the shadow occurs per 10 cu mm volume change per 5 cc of part. Table 1 indicates the necessary linear movement of the shadow for parts of various sizes. As a result of such standardization each millimeter of movement of the string shadow on the plethysmogram equals 1.0 cu mm change in volume per 5 cc of part. This eliminates calculations to reduce recordings to a common unit of measurement and permits direct and quantitative reading of the plethysmogram.

e) Taking the plethysmogram.—After standardization the plethysmogram is recorded. The string shadow should be brought to the center of the camera slit at the beginning and on termination of each recording to insure accurate measure of gross changes in volume. During recording the

calibrator lever is deflected to make an associated record of the standardization for future reference. The recording is made for 3 or 4 min (longer periods are required to study gamma deflections) at slow camera speed. If the string shadow tends to move away from the camera slit it should be returned to position. Base-line adjustments should be used as infrequently as possible. The string shadow is allowed to move back and forth spontaneously. If the subject is properly relaxed, base-line adjustments are used relatively little. It is advisable to terminate each record-

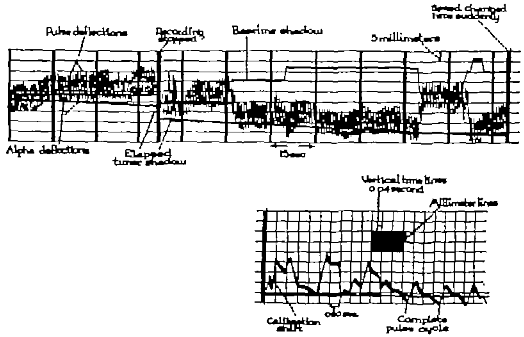

FIG. 3.—Drawing of segment of plethysmogram. Pulse and alpha deflections, the more rapid deflections, are recorded primarily by the bow string. Base-line shadow records gross changes in volume of the part, such as large beta and the gamma deflections. At fast speed, configurations of pulse waves are evident. From the time the camera was turned off the first time until it was turned on again, the elapsed timer shadow shifted 6 mm; the shift represents a 1.5 min lapse of time.

ing with a few seconds of recording at fast camera speed to permit study of morphologic characteristics of individual pulse waves. With the recording completed, set all valves to "open" before disconnecting the tubing from the extremity cup.

The plethysmogram.—This is an ordinary type of Cartesian co-ordinate. In Figure 3 the slow speed record shows the volume of the pulse wave very well without detailed configurations. The fast speed record clearly depicts details of the pulse wave. Figures 3 and 4 show the *alpha waves* or deflections and *respiratory* and *beta deflections*. Portions of gamma waves are being traced by the pin shadows of the base-line adjustment. The several types of spontaneous variations in volume of the part are shown in Figure 4. Change of the string shadow to a new position by the base-line adjustment causes a sudden simultaneous change

in position of this shadow and the pin shadow of the base-line adjustment (Fig. 3). The base-line indicator moves up when an increase in total volume of the part necessitates adjustment of the base-line, and vice versa, and draws a graph of gross change in volume of the part. The record therefore notes continuously sudden small changes (string record) and any simultaneous large changes in volume of the part (base-line indicator).

The plethysmogram is a record of the changes in volume of the part in relation to time. Volume changes for the usual periods of time of recording must be due to fluctuations in volume of at least three types of fluid:

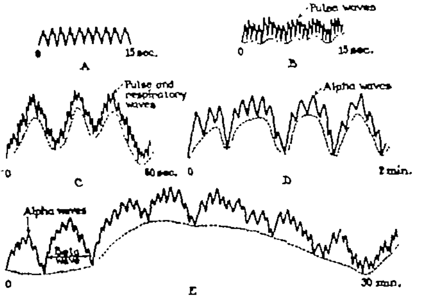

FIG. 4.—Five types of spontaneous volume deflections. (Reproduced by courtesy of Am. J. Physiol.)

blood in the vessels, inter- and intracellular fluid, and lymph in the lymphatics. In the average subject with the part at heart level it is unlikely that variations in volume of inter- and intracellular fluid contribute much to volume change except in special circumstances (7). Variations in lymph volume of the part probably do contribute to volume deflections of relatively slow frequency (7, 5). In the main, variations in blood volume in the part are responsible for changes in volume of the part. The recorded volume changes are the algebraic summation of many volume changes occurring to various degrees and directions in many different portions of the enclosed part caused by many little known phenomena. Because the role of the various types of blood vessels in production of volume changes is not known, only certain generally accepted concepts of function for each type of vessel can be applied to interpretation and application of the plethysmogram. Certain facts are sufficiently important to warrant discussion.

With a subject resting quietly in bed in a comfortable environment, at least five types of spontaneous deflections in volume of the part are observed (4–6, 10, 11). Spontaneous changes in tips of the fingers and toes range from less than 0.1 to 350 or more cu mm/5 cc of part.*

(a) Pulse waves or deflections: These are occasioned by the heart beat, and their volume varies markedly (Figs. 3 and 4). There is a definite relation between volume of the pulse and the alpha wave. Variations in volume in normal subjects are given in Table 2. Frequency of pulse deflection varies with heart rate.

TABLE 2.—Quantitative Characteristics of Pulse and Alpha Deflections of 12 Resting Normal Adults Aged 22–65 (5 Women)

	Alpha Waves, Volume, Cu Mm/5 Cc Part		Alpha Waves, Frequency, No/Min			Pulse Waves, Volume, Cu Mm/5 Cc Part		
	Mean	Max.	Mean	Max.	Min.	Mean	Max.	Min.
Finger-tip	14.5	81.0	7.0	14	2	6.9	12.4	0.9
Toe-tip	7.1	43.4	7.7	13	2	4.0	11.5	0.7
Pinna	6 6	21.0	8 0	13	2	4.1	10.5	0.9

(b) Respiratory deflections: Variations in volume occur with the respiratory cycle (Fig. 3). They are most highly developed in the pinnae and least in the toes. Rates vary with respiratory rate; in the normal subject their volume varies from less than 0.1 to 5 cu mm.

A special type of respiratory deflection occurs most prominently in the fingers and toes within a few seconds after a deep inspiration. It may present several characteristics. (1) There is a spontaneous, sudden decrease in volume, unrelated to external stimuli, varying from 5 to 105 cu mm and associated with a decrease in volume of the pulse deflections. After a few pulse beats vasodilatation begins and continues until the previous pulsatile characteristics are reached. A series of small (2–8 cu mm) alpha waves appears before vasodilatation is complete. (2) After two or more successive deep inspirations, the response diminishes until no response follows, a kind of tachyphylaxis. The shorter the interval between deep inspirations, the less the volume change. (3) Change in the pinnae is not as definite, predictable or as large as in the fingers and toes, nor is it necessarily concordant with that in the fingers and toes. There may be an increase or a decrease in volume following deep inspiration.

(c) Alpha deflections: These volume changes, next in frequency to respiratory deflections, are present in all parts of the body. They vary in frequency and size (Table 2). They are not uniform in any fashion, although contours of the deflections may be smooth. No absolute correlation between frequency and volume necessarily exists. Volume tends to vary in different individuals. The deflections do not depend on variations in arterial pressure (4, 8). Sympathectomy and sympathetic nerve

* Henceforth volume changes in a part are given as cu mm/5 cc of part.

block inhibit alpha deflections almost entirely. These deflections in fingers, toes and pinnae do not always vary concordantly (11).

(*d*) Beta *deflections*: The succession of alpha waves is superimposed on the large beta deflections (Fig. 4). Frequency is 1–2/min and size, 5–60 cu mm. Beta deflections are exhibited in all parts. Frequency and volume are totally irregular but tend to vary concordantly in fingers, toes and pinnae.

(*e*) Gamma deflections: These develop slowly and may reach extensive changes in volume (Fig. 4), from 1 to 8/hr and from 50 to 350 cu mm in size. Variation tends toward concordance in fingers, toes and pinnae, but there is not necessarily a constant relationship.

Each type of deflection is superimposed on the next slowest. At least one other deflection, of a frequency slower than that of the gamma deflection, has not had intensive study. During prolonged studies, variations in room temperature may change the volume of air in the extremity cup. To study quantitatively the deflections of slow frequency, room temperature must be controlled. Perspiration occurs at an almost uniform rate under comfortable environmental conditions and therefore shifts the base line uniformly without distorting the volume deflections.

In most circumstances pulse and alpha deflections tend to vary directly with each other (11). However, when there is marked vasoconstriction both deflections are small; with marked vasodilation the pulse deflections have large volume while the alpha deflections are small. When vascular tone in the peripheral blood vessels is at a mean level the pulse deflections are of medium size and the alpha deflections reach large proportions. In the presence of disease these reactions are disturbed.

Striking changes in pulse and alpha deflections occur in the normal individual in special circumstances. Psychic disturbances, ranging from mild discomfort to mental tension, are reflected. Measurement of the changes in character of the spontaneous volume deflections may even aid somewhat in evaluating an individual's psychic state (4, 12). The significance of even slight decrease of temperature has already been stressed. Marked changes in deflections are noted with general or local heating, the reactions often serving as a good test for organic occlusive arterial and arteriolar disease. There are typical deflection changes with deep inspiration and shift of the part being tested with reference to heart level (14). The changes following blocking of the sympathetic nerve supply to a finger- or toe-tip (13) provide a helpful test for arterial patency in occlusive arterial disease.

Comment by David I. Abramson

The plethysmograph described here is limited in its use to study of vasomotor responses in the fingers and toes, vascular beds in which specialized vessels, the arteriovenous shunts, predominate. As a result, data obtained with the apparatus cannot be utilized to elucidate the general problem of vascular reactions in the extremities. Much evidence has accumulated to indicate that in many respects

the changes observed in the distal portions of the limbs are different from those obtained in the proximal portions. Another point to be emphasized is that with the plethysmograph of Burch and Winsor little information can be derived concerning the actual rate of peripheral blood flow. The fact that rigid environmental conditions are required to obtain significant and reproducible data precludes to a great extent wide clinical use of the apparatus. It appears to have a limited but real value in study of physiologic responses in the distal portions of an extremity to various types of stimuli and in differentiating organic from spastic vascular disorders. Compared with other types of plethysmographs, it has the advantage of being readily portable.

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V. Photoelectric Plethysmography of the Skin*

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In operation of the photoelectric plethysmograph (2) light from a Mazda 222 bulb strikes, penetrates and scatters in the skin (Fig. 1). An undetermined fraction subsequently reaches the phototube. Part of the scattering in the skin is due to its blood content. If other variables which influence the amount of light reaching the phototube remain constant, changes in the blood content are reflected in the photoelectric currents.

It is obvious that these devices are not plethysmographs but photometers which may be used to follow changes in the blood content of tissues. As photometers they are influenced by variables other than the blood content. It is therefore interesting that the design of photoelectric plethysmographs followed rather than preceded that of photoelectric

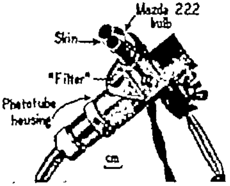

FIG. 1.—Photoelectric plethysmograph.

oximeters (6, 8). This fact may be the origin of the criticism that photoelectric records of changes in tissue opacity are affected grossly by the degree of oxygenation or reduction of hemoglobin. Direct information on the absolute magnitude of this error is not available, but the chief effect on light transmission is probably due to multiple scattering of light from the surfaces of the erythrocytes (9). If errors due to the degree of oxygenation of hemoglobin were significantly large, calibration of the photoelectric plethysmogram, described later, probably would not be possible.

Other difficulties arise from movement of the part relative to the instrument owing to muscle contractions, transmission of respiratory movements, building or stand vibrations; all produce important artifacts. Occasionally, large cardiac ballistic effects on the head preclude recording from head skin. Artifacts in skin records may occur also because changes in volume of deeper tissues can alter the position of the skin relative to the plethysmograph and so affect the amount of light reaching the

* Supported in part by grants from the American Medical Association and the United States Public Health Service.

the changes observed in the distal portions of the limbs are different from those obtained in the proximal portions. Another point to be emphasized is that with the plethysmograph of Burch and Winsor little information can be derived concerning the actual rate of peripheral blood flow. The fact that rigid environmental conditions are required to obtain significant and reproducible data precludes to a great extent wide clinical use of the apparatus. It appears to have a limited but real value in study of physiologic responses in the distal portions of an extremity to various types of stimuli and in differentiating organic from spastic vascular disorders. Compared with other types of plethysmographs, it has the advantage of being readily portable.

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Deflections of the recording galvanometers due to phototube signals vary in amplitude with light intensity, current output of the phototube, amplification and recorder sensitivity, as well as with changes in blood content. Calibration (2) of the deflection on the record (Fig. 2) in terms of changes in blood content is readily obtained by comparison with the deflection produced on insertion of a clear glass plate (section from a clear microscope slide 1.1 mm thick) in the path of light returning to the phototube (Fig. 1). This piece of glass (the *filter*) is mounted on a metal arm permanently attached to the plethysmograph. The deflection in the record produced by the filter is the filter unit. The deflection on the

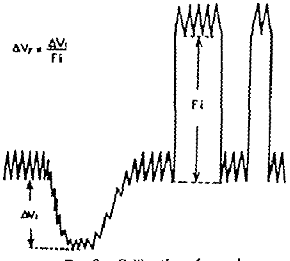

FIG. 2.—Calibration of record.

photoelectric record due to changes in blood content may then be expressed in terms of the filter unit from the equation

$$\Delta V_f = \frac{\Delta V_1}{F_i}, \qquad (1)$$

where ΔV_1 is the deflection on the record due to change in blood content, in millimeters, and F_i is the deflection due to insertion of the filter, in millimeters; ΔV_f is then given in filter units. This procedure was followed for several years until success was attained in estimating the volume equivalent of the filter unit (5). Calibration experiments with mechanical plethysmographs (5) on the finger indicate that the actual change in blood content of a particular skin area may be calculated from the photoelectric plethysmogram by application of equations (1) and (2) to the plethysmogram:

$$\Delta V = K_v \cdot \Delta V_f, \qquad (2)$$

where ΔV_f is the photoelectric deflection due to change in blood content, in filter units, and K_v is the volume equivalent of the filter unit and has the numerical value of 0.00026 cc/cm² skin/filter unit; ΔV is the change

phototube. Proper placement of the limb and contact of the plethysmograph with skin over bone are required to yield valid records.

PROCEDURE

a) Recording of photoelectric currents.—Recording devices vary from simple galvanometric arrangements to direct coupled amplifiers of special design. Amplifiers are preferred because of flexibility in recording arrangements, using high frequency galvanometers which permit the recording of volume pulses without distortion. Amplification permits use of lights of low intensity and thus decreases heating effects which may be quite significant in the presence of low blood flows.

A conveniently mounted *phototube* is readily available.* With the skin plethysmograph as usually employed, this phototube furnishes a current of about 2×10^{-8} amp which through a resistance of 1 megohm provides an input to the amplifier of 2×10^{-2} v. The superimposed signal due to changes in blood content of the finger pad is of the order of 5–20 mv. These values depend on intensity of illumination and value of resistance through which the photoelectric current flows.

Suitable *amplifier* design must meet certain requirements: quiet operation, stability, freedom from drift, linearity of response over a considerable range. The required current gain is a function of the current sensitivity of the recording galvanometer. A suitable amplifier is described by Jochim (p. 111).

Rectifiers which supply direct current with a sufficiently low ripple content are available for both filament and plate circuits, thus eliminating the need of batteries. Optically recording galvanometers may be obtained from several manufacturers. Mechanically recording galvanometers are also available but are not as adaptable to experimental arrangements. An inexpensive recorder which is quite satisfactory for qualitative purposes is made easily from a milliammeter by removing the glass case, bending the needle so that the long arm rotates in the axis of the coil and mounting a mirror on the vertical needle arm.

When one desires to record only the volume pulses (this has some advantages in qualitative study of arteriomotor reactions) one may use conventional condenser coupled circuits; however, the condenser coupling between stages should have a fairly long time constant—a condenser with a capacity of 4 microfarads operating through a grid leak of 200,000 ohms usually permits faithful reproduction of the pulse wave unless pulse rate is extremely slow.

b) Quantitative photoelectric plethysmography.—The following description is applicable only to estimation of changes in blood content in the skin with the skin plethysmograph and only when the glass filter to be described and the phototube already mentioned are used. Substitution of other phototubes and of other filters requires calibration. Applicability of similar procedures to other tissues has not been explored.

* Visitron type 51A, Rauland Corp., Chicago 41.

gree of reliability of the procedure in estimation of finger blood flow in these cases. This statement applied only to pad flows in excess of 0.1 $cc/cm^2/min$; lower flows present special technical problems in calibration. In cases in which the blood supply is furnished through multiple small collateral channels, the influence of these channels on the relation of the amplitude of the skin pulse to flow deserves recognition (1). Caution is indicated in acceptance of the quantitative significance of photoelectric criteria of flow levels in such circumstances.

Quantitative relations between changes in blood flow and in blood content (5).—In the usual vasoconstrictor responses in the finger pad, such as those elicited reflexly, a decrease in blood flow of 0.10 $cc/cm^2/min$ is accompanied by a decrease in blood content of 0.0012 cc/cm^2. Variations from this relation are relatively small in such responses. However, when the finger is exposed to cold (4 C) this relation may change grossly. Similar deviations may be observed in other skin areas. They are interpreted as indicating an "active" venous participation in the reaction. This procedure is therefore available on occasion as a means of distinguishing arteriomotor and venomotor reactions.

Estimation of blood content of minute vessels of skin.—The vasoconstriction elicited in the skin by a blast of cold air probably empties the minute vessels completely, as the photoelectric estimation of changes in the blood content of these vessels leads to values which may be approximated by other methods (5). A total blood volume of 50 cc/m^2 is estimated to be contained in the minute skin vessels under "average" conditions.

Comment by H. C. Bazett

The term plethysmograph is possibly useful with the connotation here employed, but it is apt to be misleading. The photoelectric method can at best merely measure the content, and fluctuation of content, of blood in the superficial skin, and the records must remain unaffected by variations in blood in deeper tissues. While this may be regarded as a limitation, it may also be an asset, for the commonly used plethysmographic methods give no indication of the position of the vessels involved in producing changes.

Hertzman claims that the method is not seriously impaired by errors due to variations in the degree of oxygenation. In this he is probably correct, at least under normal conditions. Blood supplying the skin undergoes little reduction in passage, since the metabolic level of the skin is low; the main function of blood flow in the skin is to control heat exchange. The oxygen content of venous blood from the superficial veins of the hand has an average saturation of 91 per cent. Probably the figure is representative for skin blood (Goldschmidt, S., and Light, A. B.: Am. J. Physiol. 73: 173, 1925). It is therefore unlikely that variations in oxygen saturation would often be a source of error. Under abnormal conditions, however, such errors might arise, and it might be wise to prevent any such possibility by utilizing filters to give a wavelength approximately equally absorbed by both oxygen and reduced hemoglobin.

Hertzman states that normal vasoconstrictor responses usually give a constant relation between the decrease in flow per minute and the decrease in blood con-

in blood content of the skin expressed as cc/cm² skin. This procedure is applicable to measurement of amplitude of the cutaneous volume pulse and of the slower changes in volume resulting from vasomotor reactions, thermal effects, etc.; it is not applicable when condenser coupled circuits are used.

c) Estimation of cutaneous blood flow from amplitude of photoelectrically recorded cutaneous volume pulses (*4*).—Calibration of the photoelectric plethysmogram of the skin in terms of changes in blood content permits estimation of the actual amplitude of the cutaneous volume pulses. In the finger pad, these vary from 0.00012 cc/cm² (constriction) to 0.002 cc/cm² (dilatation). Simultaneous measurements of blood flow in the terminal phalanx of the finger by a calorimetric method and by the technique of venous occlusion indicated a fairly linear relation between the level of blood flow and the amplitude of the cutaneous volume pulses in the flow range of 0.05–0.7 cc/cm²/min.

This observation is expressed in the equation

$$\text{blood flow} = K_f \cdot VP,$$

where VP is the amplitude of cutaneous volume pulse in cc/cm² of skin, and K_f is the flow equivalent of VP; K_f has the numerical value of 0.10 cc/cm²/min for a VP of 0.00026 cc/cm².

The quantitative relation between cutaneous blood flow and amplitude of the skin pulse is not measurably affected by variations in pulse rate within the usual resting range. It is also of interest that only about 0.15 of the flow pulse distends the minute vessels during the pulse. This indicates a very small *windkessel* effect in these vessels, an observation which may be predicted from theoretical considerations supported by other data (7, 10).

This procedure is probably applicable to skin areas other than the finger. Thus, one may estimate total cutaneous blood flow of the body by sampling the skin pulses in various areas; the resulting values are somewhat higher than those calculated from thermal data (11). This difference may be due to a possibly nonlinear relation between flow and pulse amplitude at the very low flows encountered in the resting subject in the skin of the trunk, arms and legs. Calibration experiments suitable to explore this possibility offer serious technical difficulties, but preliminary data obtained on chilled subjects at the extremely low finger flows of 0.005 cc/cm²/min indicate striking reduction in the flow equivalent of the skin pulse at such flow levels. Whether the flow equivalent of the pad pulses is transferable quantitatively to cutaneous vascular beds other than that of the finger remains to be studied. Nevertheless the data reveal important regional differences in level of cutaneous blood flow and in its variations under various circumstances.

Preliminary study of applicability of the flow equivalent of the cutaneous volume pulse in the presence of arterial disease and such mechanical factors as those operating in scleroderma (3) indicated a high de-

of one type of sectional fluid displacement plethysmograph, an instrument found to be satisfactory for quantitative estimation of arterial blood flow and changes in vasomotor tone in human physiologic studies. Other methods are described in principle only; details of apparatus and technique may be obtained from accompanying references. Plethysmographic methods for circulatory studies of the viscera in animals, although similar in general principle to the method herein described, are not included because of their highly specialized applicability.

1. *Fluid displacement plethysmography.*—The methods in widest use today employ a water-air system in which the enclosed extremity is surrounded by water and displacements of relatively small volumes of air above the water surface are recorded. It has several advantages. The water, being incompressible, adds sensitivity to the recording system. Temperature changes, which are most difficult to control in an all-air system, are readily controlled by use of suitable water-heating and -circulating devices. Systems employing only water have been described but have inherent difficulties related to control of the hydrostatic pressure effects on the enclosed limb.

Use of a sectional plethysmograph, by means of which only a segment of an extremity (hand, forearm segment or calf) is enclosed in the apparatus, has yielded much information which could not be obtained were the entire extremity studied as a unit. There exists much evidence, for example, regarding the differences in vascular responses of the hand and of the forearm. The differences in proportion of skin, subcutaneous tissue and muscle vary widely in the distal as contrasted to the proximal portion of an extremity. It would seem necessary, therefore, for accurate studies, that these portions of the peripheral circulation be studied separately. The sectional plethysmograph described here has this advantage; it is readily adaptable to circulatory studies in the hand, forearm or calf and, with slight modification in construction, can accommodate the foot.

PROCEDURE

a) *Apparatus.*—The plethysmograph *chamber* is essentially a metal box with circular openings at opposite ends through which the extremity is placed. The limb rests comfortably on an adjustable wire gauze cradle within the plethysmograph. Individually fitted, nonconstricting rubber cuffs made of thin rubber sheeting are cemented to the shaved skin of the limb at either end of the plethysmograph. The cuffs are fastened by heavy rubber bands and adjustable brass collars to projecting rims around each circular opening. The cuffs, extending from the surface of the extremity to the plethysmograph chamber, are supported externally by suitably shaped adjustable metal and celluloid diaphragms fastened to the apparatus so as to prevent distention of the rubber diaphragm. When properly adjusted the limb segment is enclosed in a water-tight, relatively nondistensible and nonconstricting chamber. For details concerning construction of the apparatus and rubber cuffs, see Abramson (1).

tent, the former having a numerical value some 83 times the latter. When the finger is exposed to extreme cold (4 C) this proportion may be grossly changed, and the alteration is interpreted as implying "active" venous participation in the response to cold. Presumably this means that the change in blood content is unusually large and that the ratio is reduced from 83:1 to some lower value. It would be useful if this direction and magnitude of the change were indicated.

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VI. Fluid Displacement and Pressure Plethysmography

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Plethysmography or volume recording has become increasingly useful since Brodie and Russell (4) in 1905 established the principles on which are based the venous occlusion method of determining blood flow. Applications of these principles have covered a wide field; volume measuring methods have been applied to the viscera as well as to the extremities in man and animals (1, 3, 6, 8–10, 13, 14, 16). The most productive applications have been in peripheral circulatory studies. Suitable apparatus have permitted estimation of arterial blood flow, study of variations in vasomotor tone and quantitative measurement of changes in extravascular fluid. Plethysmographic methods for obtaining data on mean capillary blood pressure and dynamic vascular volume have been described (15).

Because of increasing clinical interest in peripheral circulation measurements in human extremities, this discussion includes in detail the use

of one type of sectional fluid displacement plethysmograph, an instrument found to be satisfactory for quantitative estimation of arterial blood flow and changes in vasomotor tone in human physiologic studies. Other methods are described in principle only; details of apparatus and technique may be obtained from accompanying references. Plethysmographic methods for circulatory studies of the viscera in animals, although similar in general principle to the method herein described, are not included because of their highly specialized applicability.

1. *Fluid displacement plethysmography.*—The methods in widest use today employ a water-air system in which the enclosed extremity is surrounded by water and displacements of relatively small volumes of air above the water surface are recorded. It has several advantages. The water, being incompressible, adds sensitivity to the recording system. Temperature changes, which are most difficult to control in an all-air system, are readily controlled by use of suitable water-heating and -circulating devices. Systems employing only water have been described but have inherent difficulties related to control of the hydrostatic pressure effects on the enclosed limb.

Use of a sectional plethysmograph, by means of which only a segment of an extremity (hand, forearm segment or calf) is enclosed in the apparatus, has yielded much information which could not be obtained were the entire extremity studied as a unit. There exists much evidence, for example, regarding the differences in vascular responses of the hand and of the forearm. The differences in proportion of skin, subcutaneous tissue and muscle vary widely in the distal as contrasted to the proximal portion of an extremity. It would seem necessary, therefore, for accurate studies, that these portions of the peripheral circulation be studied separately. The sectional plethysmograph described here has this advantage; it is readily adaptable to circulatory studies in the hand, forearm or calf and, with slight modification in construction, can accommodate the foot.

PROCEDURE

a) *Apparatus.*—The plethysmograph *chamber* is essentially a metal box with circular openings at opposite ends through which the extremity is placed. The limb rests comfortably on an adjustable wire gauze cradle within the plethysmograph. Individually fitted, nonconstricting rubber cuffs made of thin rubber sheeting are cemented to the shaved skin of the limb at either end of the plethysmograph. The cuffs are fastened by heavy rubber bands and adjustable brass collars to projecting rims around each circular opening. The cuffs, extending from the surface of the extremity to the plethysmograph chamber, are supported externally by suitably shaped adjustable metal and celluloid diaphragms fastened to the apparatus so as to prevent distention of the rubber diaphragm. When properly adjusted the limb segment is enclosed in a water-tight, relatively nondistensible and nonconstricting chamber. For details concerning construction of the apparatus and rubber cuffs, see Abramson (1).

The apparatus described here* consists of a metal chamber $9 \times 9\frac{1}{2} \times 4\frac{1}{2}$ in. with an enclosed heating element and motor-driven water circulator. At either end is a circular opening $6\frac{1}{2}$ in. in diameter with a projecting metal rim 1 in. wide. Each rim is fitted with an adjustable brass tension collar by means of which water-tight seals can be made with a rubber diaphragm or sleeve in place. Use of the individually fitted rubber diaphragms already referred to is feasible in the hands of many workers; the occurrence of water leaks, however, probably accounts for the reluctance of some investigators to use this type of apparatus. Use of a rubber sleeve, if made of suitably thin rubber sheeting and of proper dimensions, has proved satisfactory. The sleeve is constructed by cutting a rectangle 14×21 in. of rubber sheeting 0.002 in. thick.† The long edges are cemented together in a double hem with neoprene adhesive. A sleeve is thus formed with a diameter approximately $6\frac{1}{2}$ in., each end of which can be permanently sealed around the projecting rims of the plethysmograph. If the rubber is sufficiently thin and the extremity well dried with talc, the abundant folds of rubber will lie against the surface of the limb at all points when the apparatus is filled with water. Supporting diaphragms of celluloid and metal are adjusted to each end of the plethysmograph and prevent protrusion of any rubber folds. Diaphragms are cut from rectangular pieces (6×9 in.) of celluloid $\frac{1}{32}$ in. thick. Longitudinal slots perpendicular to the long edge on one side allow the diaphragms to slide horizontally on the threaded supporting rods projecting from each end of the metal box. Semicircular or semi-elliptical sections removed from the central edge of each piece enable a pair of diaphragms to encircle accurately the proximal or distal end of the limb segment within the plethysmograph. An assortment of paired diaphragms forming circular or elliptical openings ranging from 2 to 7 in. in diameter in $\frac{1}{2}$ in. gradations is adequate for most studies.

Many types of *recording apparatus* have been described which give satisfactory results. Both photographic and direct writing methods are feasible. For direct recording of volume changes, a miniature Krogh spirometer or Brodie bellows connected to the air layer in the plethysmograph is satisfactory. A 3-way valve between plethysmograph and recording system allows disconnection of the recording system during adjustment. The Brodie bellows has greater accuracy and sensitivity; a bellows of about 30 ml capacity can be constructed from goldbeater's membrane and a hinged aluminum frame. The membrane is kept pliable by application of glycerin to both surfaces. A writing lever about 20 cm long gives satisfactory magnification for recording with either smoked paper or an ink-writing pen on ordinary paper. An electrically heated writing lever using heat-sensitive paper‡ is most satisfactory for clinical work.

* Manufactured by George H. Wahmann Mfg. Co., Baltimore.

† Rubber sheeting of suitable thickness has been obtained from Dewey and Almy Chemical Company, Cambridge, Mass.

‡ Permapaper (unprinted), supplied by Sanborn Instrument Co., Cambridge, Mass.

Two pressure cuffs are used when blood flow to the forearm or calf is measured. A narrow cuff 3 in. wide is placed around the wrist or ankle and by means of a 3-way valve is connected to a 20 liter air pressure reservoir maintained at 50 mm Hg above systolic blood pressure. A second cuff 5 in. wide and long enough to encircle the limb $1^1/_2$ times is applied just above the elbow or knee. This is connected by a 3-way valve to a 20 liter air pressure reservoir maintained at 30–40 mm Hg below the subject's diastolic pressure. The pressure reservoirs are connected by tubing sufficiently wide to permit sudden inflation of the cuffs.

b) *Technique.*—Resting blood flows are most often determined with the subject recumbent and the enclosed limb segment at heart level. The forearm or calf segment is powdered liberally and placed in the plethysmograph. The folds of the rubber sleeve are arranged uniformly along the limb segment in the apparatus. The extremity is supported by sandbags on either side of the plethysmograph so that the limb rests lightly in the apparatus without compression. Celluloid diaphragms of suitable size are placed on the threaded supports at the forward end of the metal box and moved centrally so as to encircle the limb completely, leaving a 1–2 mm space on all sides. Two metal diaphragms are then placed externally and adjusted in a vertical direction encircling the limb. These support the more accurately fitting celluloid diaphragms. The diaphragms are held firm by tightening thumb screws on each threaded supporting rod. Similarly, a pair of celluloid and metal diaphragms is adjusted to the proximal end of the plethysmograph, carefully avoiding constriction. The narrow occluding cuff is placed around the wrist or ankle and the proximal or collecting cuff is applied loosely just above the elbow or knee.

Water at the desired temperature is placed in the plethysmograph chamber until it reaches a fixed level several centimeters above the upper surface of the enclosed limb segment, causing the abundant folds of the thin rubber sleeve to lie against the surface of the extremity at all points. By means of an electric heater in the chamber and a motor-driven circulating fan, water temperature is kept relatively constant. Room temperature is recorded and kept at desired levels throughout a determination.

When the instrument is properly adjusted to the subject the apparatus is calibrated by successive additions of 1 ml of water to the plethysmograph. A Cornwall syringe connected by a 2-way valve to a small water reservoir permits convenient introduction of measured quantities of water through the outlet at the base of the plethysmograph chamber. Deflection of 0.5–1.0 cm of the writing lever for each additional milliliter of fluid introduced is satisfactory.

Before determinations are recorded the apparatus is checked for evidence of venous constriction at the proximal opening of the plethysmograph by inflating the congesting cuff to 10–15 mm Hg. A definite increase in volume while the cuff is inflated and a drop in volume with re-

lease of pressure is recorded if no appreciable constriction is present. If pressures higher than 15 mm Hg are required to produce this change, significant venous obstruction can be assumed to be present and readjustment of the proximal rubber cuff or diaphragm may be necessary.

A standard rest period of 30–60 min with the subject in position permits vascular adjustments to come to an equilibrium. With the apparatus properly adjusted and the subject cautioned to remain quiet, variations in volume caused by changes in vasomotor tone are best recorded on a slowly moving kymograph. Rhythmic variations in volume can be identified as being due to (*a*) arterial pulsations, (*b*) respiratory waves or (c) spontaneous changes in vasomotor tone. Volume changes due to arterial pulsations are easily recognized and are always observed if the apparatus is properly adjusted. Changes synchronous with respiration are common in the upper extremity and are largely mechanical artifacts caused by respiratory motions of the chest transmitted to the arm. These waves can often be eliminated by placing the extremity in greater abduction. The slower, more irregular variations in volume are due to changes in vasomotor tone and are more pronounced in the digits and distal portions of the extremity than in a proximal segment. They become more evident at moderately elevated temperatures and diminish with cold. Sleep, mental or emotional stimulation and pain affect these spontaneous variations.

Arterial blood flow determinations are made by sudden occlusion of venous return from the enclosed segment. The subsequent increase in volume of the distal portion of the extremity during the first few seconds following occlusion is presumed to be due entirely to the unrestricted arterial inflow. Several obvious precautions should be taken. Pressure level of the venous occlusion cuff must be set high enough to prevent any venous outflow but not to interfere with arterial circulation. Pressure 30–40 mm Hg below the subject's diastolic pressure gives consistently satisfactory records. During the determination of blood flow in a proximal segment of an extremity, it is necessary to occlude the circulation to more distal portions. Venous return from the hand, for example, introduces a source of error when the forearm segment is being studied. The distal pressure cuff, therefore, is inflated to approximately 50 mm Hg above systolic pressure about 30 sec before the forearm blood flow is recorded and kept inflated for several minutes during a series of determinations. At least 30 sec is necessary after inflation of the distal cuff to allow for readjustments in vascular tone in the proximal segment of the extremity.

To measure blood flow in the hand the sectional plethysmograph can be modified by removing the sleeve and closing one of the circular openings with a water-tight rubber diaphragm supported by a rigid metal plate fastened externally to the chamber. A nonconstricting rubber cuff is cemented to the wrist and the hand allowed to rest comfortably on the wire cradle in the plethysmograph. In a manner similar to that already

described, the rubber cuff is fastened to the projecting rim of the circular opening and a water-tight seal formed by rubber bands and brass tension collar. A collecting pressure cuff is placed immediately proximal to the enclosed portion of the extremity and connected to an air pressure reservoir maintained 30–40 mm Hg below diastolic pressure. In other respects the technique is similar to that described for the forearm.

c) *Interpretation.*—Sample tracings taken from a normal calf segment at the indicated temperatures are shown in Figure 1. The upper line

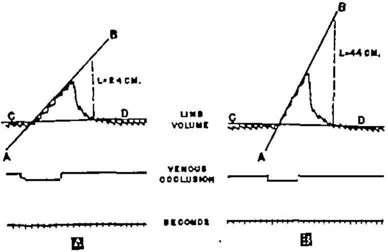

FIG. 1.—Sample tracings of fluid displacement plethysmograph, and calculations. *A: calf segment volume, 630 cc; water temperature, 37 C; calibration factor, 0.5* cm/ml.

$$\text{blood flow} = \frac{600 \times 2.4}{0.5 \times 630} = 4.6 \text{ ml/100 cc tissue/min.}$$

B: calf segment volume, 630 cc; water temperature, 43 C; calibration factor, 0.5 cm/ml.

$$\text{blood flow} = \frac{600 \times 4.4}{0.5 \times 630} = 8.4 \text{ ml/100 cc tissue/min.}$$

represents limb volume; the signal mark on the center line designates onset and release of venous occlusion, and the lower line indicates time in seconds. Arterial inflow is calculated from the slope of a line (A–B) drawn through corresponding points on the first two or three pulse waves following venous occlusion. The base line (C–D) from which the slope is derived is obtained by connecting corresponding points on the pulse waves immediately preceding venous occlusion. From the time scale, a distance equivalent to 10 sec is measured off on the base line and a perpendicular (L) erected at this point. Blood flow in ml/min/100 cc of tissue is calculated from the formula:

$$\frac{600 \times L}{K \times V} = \text{blood flow (ml/min/100 cc tissue)},$$

where L is the perpendicular rise in slope during 10 sec, in centimeters; K, calibration constant of the recording apparatus, in cm deflection/cu cm change in volume; V, volume of the enclosed limb segment, in cubic centimeters. Volume of the extremity can be measured by suitable water displacement methods in a graduated cylinder; for forearm or calf, volume can be mathematically calculated from the average circumference and length of the segment.

Reliability of the method depends largely on proper construction of the slope and base lines. Several artifacts may cause difficulty in analysis of the record. Obviously, the system must be water-tight. A persistent slow drop in the base line may be due to leakage and must be corrected. Frequently, a sudden sharp rise or drop is observed in the volume recording coincident with inflation of the congesting cuff. This is a mechanical artifact due to shifting of the extremity with expansion of the cuff. Reapplication of the cuff and readjustment of the supporting sandbags minimize this artifact. If recognized and consistent, the base line artifact can be ignored and the slope constructed as before.

From mathematical considerations it is readily seen that very slow or rapid rates of flow may give inflow curves whose slopes approach either 0° or 90° with respect to the base line. Calculations based on curves in these extreme regions are inaccurate. It is useful, therefore, whenever possible to adjust the speed of the kymograph to allow inflow curves whose slopes are between 30 and 60°. A kymograph paper speed as low as 1–2 mm/sec may be necessary in very slow rates of flow, whereas speeds as high as 5–6 mm/sec are used in more rapid blood flow rates.

Errors in calculation may arise from nonlinearity of the recording system. The writing lever inscribes an arc, whereas the calculations are based on rectilinear rise from the base line. If the lever is sufficiently long, however, and the inflow curves are obtained in the same region of the recording arc, the error is minimized and may be disregarded. Calibration of the bellows before each series of determinations with the extremity in position gives deflections so close to linearity that correction for the arc would not appear to be necessary. Methods have been described, however, using special writing levers (19) or optical recording systems (6) which eliminate the nonlinearity of a simple writing lever.

d) Applications.—Plethysmographic methods have contributed much to the understanding of factors concerned in normal peripheral circulation in man. The technique has been applied to study of vascular responses to chemical and physical stimuli in normal (12) and hypertensive states (17, 18) and in functional and organic vascular disorders (11) and is useful in evaluation of the vascular effects of drugs (16) and other therapeutic procedures (2, 7). The chief disadvantage of plethysmography lies in the many inherent technical difficulties which have prevented the widespread application it deserves.

2. *Pressure plethysmography.*—For measuring the relatively small variations in extravascular tissue fluid the usual plethysmograph is not

satisfactory. A pressure plethysmograph (10, 13) has been devised in which pressure is exerted on the surface of the forearm to collapse the blood vessels before volume of the segment is determined. The state of contraction or dilatation of the blood vessels does not interfere with measurement of relatively small changes in volume of extravascular tissue fluid. By appropriate calculations the number of milliliters of fluid filtered or absorbed per 100 cc of forearm tissue can be determined with reasonable accuracy.

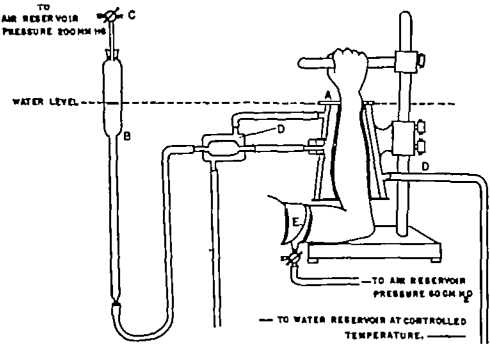

FIG. 2.—Pressure plethysmograph. *A*, metal plethysmograph with inner rubber sleeve; *B*, 200 ml buret; *C*, valve to pressure reservoir; *D*, circulating water at controlled temperature; *E*, congesting pressure cuff.

PROCEDURE

a) *Apparatus*.—The apparatus (Fig. 2) consists of a water-filled metal cylinder with an inner rubber sleeve connected by pressure tubing to a 200 ml buret. By opening a valve to an air pressure reservoir, 200 mm Hg of pressure is applied to the water in the buret and transmitted to the plethysmograph, thereby compressing the enclosed forearm segment. As water flows out of the buret during application of pressure, the buret is raised and volume readings are made with the water level in the buret at the same height as the top of the plethysmograph. Readings are made at the end of 2 min of compression, then pressure is released. The volume thus recorded has been called the "reduced" or "avascular" arm volume. Temperature is controlled by circulating water from a reservoir kept at constant temperature through a jacket around the plethysmograph cylinder and inflow tube.

b) Technique.—The subject remains recumbent with the arm abducted and elbow flexed for 1 hr before observations begin in order to control variations in the amount of extravascular fluid caused by previous activity or dependency. Readings are made at 10 min intervals. Difference in volume between successive readings of the compressed segment represents either changes in extravascular fluid or artifact due to small amounts of tissue expressed from the instrument during each period of compression. Brown, Wise and Wheeler (5) found that with each subject tested a small constant decrease in reduced arm volume at 34–35 C could be attributed to the mechanical artifact of each compression. An increase in volume can be ascribed to capillary filtration, and a decrease in volume greater than that due to the mechanical artifact can be ascribed to capillary absorption or lymphatic drainage. Filtration rates can be computed and expressed as ml of fluid/100 cc of forearm tissue/min/cm rise in venous pressure.

c) Application.—This method is applicable to studies in which fluid movement in the tissues of the extremities warrants quantitative measurement. Due care in eliminating constriction at the proximal opening of the plethysmograph and proper preparation of the subject before observation are essential for reliable results. In patients with edema, standardization of water and salt intake and elimination of gravitational effects on fluid accumulation in the observed forearm are probably necessary for consistent results. Although pressure plethysmography has received relatively little attention, it is described here because, properly applied, it may give extensive information concerning tissue fluid disturbances in many clinical conditions.

NOTE.—This section was reviewed by Eugene M. Landis.

Comment by Harold D. Green

An optically recording plethysmograph has been described by G. W. Wright, and K. Phelps (*Comparison of procedures for increasing blood flow to limbs using an improved optical plethysmograph*, J. Clin. Investigation 19: 273, 1940).

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VII. Measurement of Renal Blood Flow

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Measurement of renal blood flow is accomplished by *direct* methods in animals through cannulation of renal vein or artery and use of suitable flow meters, or by *indirect* methods employing the renal clearance principle, applicable to both animals and man. In the latter, almost complete extraction by the kidney of certain substances (e.g., diodrast or para-aminohippuric acid) from renal plasma makes their clearance a measure of renal plasma flow and, combined with simultaneous hematocrit determination, a measure of renal blood flow.

1. *Direct methods.*—(*a*) *Venous outflow.*—Since direct cannulation of the renal vein may necessitate undesirable stoppage of renal circulation and because the fragile character of the renal vein tissue does not lend itself well to direct cannulation, indirect approach via the external jugu-

lar vein and vena cava employing a long cannula is recommended. A cannula suitable for use in dogs is constructed from thin-walled brass tubing of 0.5 cm inside diameter. It should be about 40 cm long and slightly bent at the tip, which has several large perforations. A small elevated collar 1 cm from the tip insures secure ligation. The cannula is introduced into the right jugular vein, with a slow stream of saline perfusing it, and is gently passed into the abdominal vena cava to the junction of the left renal vein, as visualized through a ventral abdominal incision of the anesthetized animal (30 mg/kg of pentobarbital sodium intravenously is preferred). The animal is heparinized with a priming dose of 4 mg/kg of body weight, with additional doses of 5 mg every 30 min thereafter (Connaught Laboratories heparin is used). The cannula is then introduced into the renal vein and securely ligated. Spermatic or

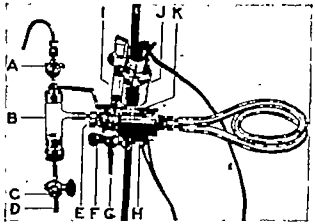

FIG. 1.—Modified bubble flow meter utilizing photoelectric cell principle. *A*, air outlet; *B*, bubble trap; *C*, coupling for renal arterial pressure manometer; *D*, outlet to renal cannula; *E*, coupling for carotid inflow cannula; *F*, 3-way valve for air injector; *G*, connection for air pressure system; *H*, photoelectric cell; *I*, air injection syringe; *J*, lamp; *K*, slot for light transmission.

ovarian veins should be ligated. Renal vein blood is directed from the renal cannula through a direct external circuit to the opposite (left) external jugular vein. A shunting key is arranged so that when depressed, the return circuit to the opposite jugular vein is occluded while simultaneously a shunt circuit is opened to allow the blood to flow into 10 ml graduated cylinders. (Alexander's method employing a strain gauge for measurement of venous outflow [this volume, p. 75] is well adapted for this purpose.) Depression of the key also actuates through a mercury contact a signal magnet in conjunction with a suitable chronometer, recording the period of outflow on the kymograph. The outflow orifice of the shunt circuit should be placed at a height equal to the pressure in the renal vein circuit. This can be approximated from the level of blood in a reservoir through which renal vein blood aliquots are reinfused. The reservior is connected by a T-cannula to the renal vein circuit at the point of return into the left jugular vein.

The length of the renal cannula creates some resistance to blood flow, and should be considered when interpreting the observations. However, renal blood flow measured in this fashion agrees well with other direct methods and with estimates derived from renal clearances (10).

b) *Arterial inflow.*—Accessibility of the renal artery by the dorsal retroperitoneal approach makes it favorable for direct measurement of arterial inflow. Use of the thermostromuhr eliminates the necessity of arterial incision, but direct cannulation can be accomplished with ease and makes preferable the use of other flow meters, e.g., the rotameter (p. 96), orifice meter (p. 102) or bubble flow meter (p. 80).

A modification of the bubble flow meter has been used advantageously, particularly when rapidly repeated measurement of mean renal blood

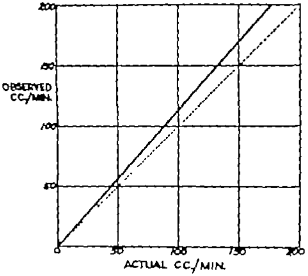

FIG. 2.—Relation of observed blood flow, as determined by rate of bubble movement (solid line), to actual direct blood flow. Dotted line indicates perfect correlation.

flow was desired, as during nerve stimulation or action of rapidly acting vasomotor drugs. In this modification a photoelectric cell records time of passage of the bubble through the flow meter circuit, permitting accurate calculation simultaneous with blood pressure measurements on the photokymograph. The principle is apparent from Figure 1. Arterial blood from a carotid artery of the heparinized animal is perfused through the flow meter directly into the peripheral renal artery through an L-cannula of suitable dimensions. The central end of the renal artery must be ligated at its junction with the abdominal aorta just before cannulation. The main circuit of the flow meter consists of lucite (plexiglas) tubing of 3 mm internal diameter and 60 cm length. Blood enters and leaves the tubing through a lucite block through which the bore of the tubing is continuous. A metal plate containing two slots directly overly-

ing the continued bore of the tubing covers the lucite block. Through the slots, light from a 6 v auto lamp is transmitted to an underlying photoelectric cell. Air from a reservoir under pressure somewhat higher than the carotid blood pressure is injected in volumes of about 0.3 cc into the inflow limb of the flow meter. Passage of this bubble under the "entering" and "exit" slots suddenly transmits light to the photocell. This impulse is amplified by a GE Victor electrocardiograph, causing the mirror of an auxiliary galvanometer of the torsion string type to register a deflection of a light beam.

To calculate volumetric flow, the exact volume of the flow meter from midpoint to midpoint of the "entering" and "exit" slots must be known. Time of passage is obtained from the electrical timing device of the photokymograph. Because the air bubble travels somewhat faster than the total column of blood, the observed flow is faster than the simultaneously measured volumetric outflow. This is a linear relationship directly proportional to the rate of flow and total length of the flow meter circuit, hence a calibration curve (Fig. 2) must be constructed by making simultaneous direct flow determinations with bubble flow at different rates.

The blood meets resistance to flow in the meter circuit, causing renal arterial perfusion pressure to be less than mean systemic arterial pressure. The pressure drop in the bubble flow meter used here is 15.0 mm Hg at a flow of 100 ml/min.

No provision has been made for constant temperature regulation of the flow meter. Because of the rapid flow, there is little temperature drop across the meter, and rectal temperature is maintained by use of a warming board.

2. *Indirect methods.*—The high extraction ratio $\left(\frac{A - V}{V}\right)$ of diodrast and para-aminohippuric acid (a value of 0.85–0.93) at arterial plasma concentrations below about 5 mg per cent means that the renal clearance $\left(\frac{UV}{P}\right)$ of these substances approaches total renal plasma flow. In fact, comparison of clearance of para-aminohippuric acid with direct plasma flow (10) has given a value of about 90 per cent of direct flow, the "effective" renal plasma flow; presumably the other 10 per cent goes to nonexcretory tissue.

When measurement of effective renal plasma flow is combined with clearance of substances which measure glomerular filtration rate, the resulting ratio, $\frac{\text{glomerular filtration rate}}{\text{effective plasma flow}}$, the filtration fraction, is valuable for drawing inferences as to the vasomotor state of the afferent and efferent arterioles. The plasma clearances of inulin, mannitol and thiosulfate are commonly used to measure glomerular filtration rate in man and, in addition, creatinine in the dog and certain other animals. Because

para-aminohippuric acid (given as the sodium salt) is well adapted to measurement of effective renal plasma flow because of ease and reliability of chemical analysis (11), and since clearance of inulin in man and of creatinine in the dog have become standard measures of glomerular filtration rate, this discussion emphasizes the use of these substances.

PROCEDURES FOR RENAL CLEARANCE

a) *Administration of infusion fluids.*—For human subjects, infusion rate from an overhanging Kelly infusion bottle is regulated by a special tunnel clamp* (2) which exerts uniform compression over the infusion tubing for 4 in. Rate of inflow is determined by counting drops through a dropper arrangement (Murphy drip) in the infusion circuit. The tubing is freed of air by rinsing with hot saline. Infusion is into the antecubital vein. Priming doses of the substances to be cleared are injected via the infusion tubing about 20 min before beginning urine collection.

For infusion into dogs, the method used in the laboratory of Dr. Homer W. Smith, New York University, is recommended. A constant rate of inflow of infusion fluid under pressure is obtained from a side-arm flask (250 ml) by directing a fine stream of mercury into the stoppered flask through a capillary tip from an overhanging Kelly infusion bottle. The capillary tip is made by heat fusion of the end of a thick-walled pipet, then reground carefully until the tip is barely patent. Rates of inflow of less than 2 ml/min are easily obtainable. Infusion is into the marginal ear vein or the cephalic vein of the forelimb.

For effective plasma flow and glomerular filtration rate of normals, the following dosages should be approximately correct. (1) Priming: in man—30 ml of 10 per cent inulin, and 5 ml of 20 per cent para-aminohippurate,† boiled for 5 min; in dogs—0.1 g of para-aminohippurate, 0.5 g of creatinine in 25 ml of saline. (2) Sustaining: in man—70 ml of 10 per cent inulin, and 20 ml of 20 per cent hippurate, diluted to 500 ml in saline, boiled for 5 min, infused at the rate of 4 ml/min; in dogs—0.5 g of hippurate and 3.5 g of creatinine in 250 ml of saline, infused at the rate of 1.5 ml/min.

b) *Collection of urine and blood.*—Goldring and Chasis (7) recommend urine collection in human subjects with an inlying 5-eyed rubber catheter, the urines being allowed to drain into a narrow-necked flask over about 15 min, following an initial discard period. Toward the close of the period, air is blown into the bladder from a 25 ml syringe and the last of the urine is removed by suction. This is followed by "washing" of the bladder with an accurately measured volume of sterile saline via the syringe; this volume at least must be recovered to conclude the period. Essentially the same technique may be used in dogs; a 2-holed, no. 14 or

* Obtainable from Harvard Apparatus Company, Dover, Mass.

† These substances are commercially prepared in these concentrations in sterile ampules: hippurate, Sharp & Dohme, Philadelphia; inulin, U.S. Standard Products, Woodworth, Wis., and Warner Institute, 113 West 18th St., New York.

16 catheter (Ameran-Acmi; Bard), used in female dogs, is best. Gentle compression on the lower part of the abdomen aids in recovery of bladder urine.

In man, 20 ml blood volume samples are drawn from an antecubital vein during the urine collection period. In dogs, 10 ml volumes are drawn from the external jugular vein with the dog supine. Heparin or saturated oxalate solution, 1 or 2 drops in the syringe and 1 or 2 drops in the blood receptacle, serves as anticoagulant. When clearances are being calculated, unless blood levels of infused substances are constant, concentrations should be plotted against time on semilogarithmic paper, and a 2 min correction allowed for passage of the substance from the blood to the bladder.

c) *Analytical methods.*—(1) Preparation of plasma filtrates. The $CdSO_4$ method of Fujita and Iwatake (5) gives a filtrate satisfactory for analysis of the commonly used clearance substances.

(*a*) Reagents: Acid cadmium sulfate—17.34 g of $3\ CdSO_4 \cdot 8\ H_2O$ and 84.55 ml of 1N H_2SO_4, q.s. 500 ml 1.1N NaOH.

(*b*) Procedure: To 9 ml of acid $CdSO_4$ and 30 ml of distilled water, add 3 ml of plasma; mix, then add 3 ml of 1.1N NaOH. Stopper the receptacle and shake well. After 10 min, centrifuge and filter through Whatman no. 1 or 2 paper (1:15 dilution). For inulin, plasma must be yeasted before analysis (see below). Also for inulin, 1.0N NaOH is substituted for 1.1N NaOH with the same cadmium mixture to avoid deficient recoveries.

(2) Determination of para-aminohippuric acid (3, 7, 11).

(*a*) Reagents: *100 mg per cent sodium nitrite* (keep only 2–3 days). 500 mg per cent ammonium sulfamate (prepared every 2 weeks). 100 mg per cent N-(1-naphthyl) ethylenediamine dihydrochloride (keep in dark bottle). 1.2N HCl.

(*b*) Procedure: Urines are diluted to an optimal colorimeter range as *follows:*

$$\frac{(\text{expected plasma level mg\%}) \times (\text{expected clearance ml/min} + 0.153)}{(\text{volume of urine ml/min}) \times (\text{washout dilution})}$$

For color reaction, use 10 ml of plasma filtrate or diluted urine. Add 2 ml of *1.2N HCl and mix. Add 1 ml of* $NaNO_2$, shake vigorously and let stand 3–5 min. Add 1 ml of ammonium sulfamate, shake vigorously and let stand 2–5 min. Add 1 ml of N-(1-naphthyl) ethylenediamine dihydrochloride, mix contents and let stand at least 10 min (out of direct sunlight). Duplicate samples are read in the Evelyn photoelectric colorimeter with filter 540. A reagent blank containing 10 ml of distilled water is set at 100 on the galvanometer. Milligrams per cent of para-aminohippuric acid is read from a standard curve plotted from known concentrations of the acid.

In human subjects it is advisable to test control plasma for chromogenic blank before clearance is begun. For this, 8 ml of the control plasma filtrate plus 2 ml of 0.2 mg per cent standard solution of para-amino-

hippuric acid is compared with 8 ml of distilled water plus 2 ml of the standard, as above. The blank value is the difference between the readings, with correction for dilution (1:1.25).

(3) Determination of creatinine (4).

(*a*) Reagents: Saturated picric acid solution. 2.5N NaOH. Prepare sufficient volume of alkaline picrate solution, using the following proportions: 5 parts saturated picric acid solution, 1 part 2.5N NaOH and 6 parts distilled water.

(*b*) Procedure: Urine is diluted to an approximate U/P ratio of 1.0. To 5 ml volumes of plasma filtrate or diluted urine add 10 ml of alkaline picrate solution. Let stand 10 min and read in the Evelyn colorimeter with filter 520. A blank containing 5 ml of distilled water and 10 ml of alkaline picrate is set at 100 of the galvanometer. Milligrams per cent of creatinine is read from a standard curve plotted from known concentrations of creatinine.

Excess cadmium may be precipitated from the plasma filtrate when alkaline picrate is added. The solution is then filtered through washed cotton directly into the colorimeter tubes for reading.

(4) Determination of inulin (7, 8).

(*a*) Reagents: Diphenylamine reagent (diphenylamine must be recrystallized from hot 70 per cent alcohol until white)—dissolve 18 g of $(C_6H_5)_2NH$ slowly in 600 ml of glacial acetic acid; add 360 ml of concentrated HCl. Yeast suspension—approximately 20 per cent made from bakers' starch-free yeast, washed in distilled water five or six times; wash again on day of use, and determine concentration accurately in Wintrobe hematocrit tubes.

(*b*) Yeasting and precipitation: Add 2 ml of diluted plasma (1:2) to 6 ml of 20 per cent yeast in a 150 × 20 mm heavy-walled pyrex tube and mix by inverting; let samples stand 15 min and shake at least three times. Then centrifuge for 15 min and remove supernatant fluid. Add 4 ml of yeasted plasma to 6 ml of cadmium sulfate in a 50 ml Erlenmeyer flask and mix; then add 2 ml of 1.0N NaOH, stopper the flask and shake well; shake occasionally again during 10 min, then centrifuge. Filter supernatant fluid through pledgets of washed absorbent cotton firmly fixed in the mouth of funnels. (Total dilution is 1:20.4, if yeast suspension is exactly 20 per cent.)

An inulin blank is determined on a sample of blood drawn before infusion is begun. The blank is determined additively as follows. In one tube, add 2 ml of plasma to 6 ml of 20 per cent yeast. In another tube, add 2 ml of distilled water to 6 ml of 20 per cent yeast suspension. To each tube add 2 ml of 20 mg per cent inulin standard; the tubes are then yeasted and precipitated as described above. The blank is the difference between the two determinations.

Urine is diluted to a U/P ratio of 1.0 for analysis. If this dilution is high, the blank is negligible. If glucose is present, the urine is treated as the plasma by yeasting and precipitating. Since most of the blank comes

from yeasting, if urines are yeasted after dilution the urine blank must be determined as described for plasma.

(c) Colorimetric procedure: Pipet 3 ml of the above filtrates or diluted urines into pyrex tubes (200 × 25 mm) and add 10 ml of diphenylamine reagent. Mix thoroughly, cover the tube with a glass tear and heat in a boiling water bath for exactly 30 min. All samples are run in duplicate. Two or three water reagent blanks containing 3 ml of distilled water and 10 ml of diphenylamine reagent, and two standards in duplicate containing 3 ml of 1.0 and 2.0 mg per cent inulin with 10 ml of diphenylamine reagent, are heated simultaneously. Cool the tubes in a water bath not below 25 C for 2 min, then read in the photocolorimeter with a 540 filter, the galvanometer being set at 100 by means of the water blanks. A standard curve for the colorimeter is made with concentrations ranging from 0.5 to 2.0 mg per cent of inulin.

(5) Determination of other clearance substances. Lack of space prohibits detailed description of analytical techniques of other substances important in indirect measurement of renal blood flow. For diodrast, see references (1) and (7); for mannitol, (7) and (12); for thiosulfate, (6) and (9).

NOTE.—This section was reviewed by Homer W. Smith.

Comment by J. Maxwell Little

For the Evelyn colorimeter the band widths for the recommended filters are: 540 mμ (515–570), 520 mμ (495–550).

In the determination of para-aminohippuric acid, the procedure can be shortened by treating a control plasma in the same manner as an unknown. The galvanometer is set on 100 per cent transmission, using the control plasma preparation. In this way the control plasma chromogenic material is compensated for without need of the indirect approach suggested in the procedure outlined.

Ammonium sulfamate may be purchased from La Motte Chemical Products Co., Baltimore; and N-(1-naphthyl)-ethylenediamine from the same company or from Eastman Kodak Company, Rochester, N. Y.

Comment by Harold D. Green

It should be noted that if any desired concentration of the test substance may be attained, the solution should be adjusted so that the resulting concentration gives a galvanometer reading between 10 and 70 per cent transmission. The greatest percentile accuracy is attained at about 40–50 per cent transmission.

It is possible that the method described here does not result in complete isolation of the kidney. In this laboratory we have successfully perfused the completely isolated kidney without at any time interrupting the blood flow (see p. 120).

Dock (Ann. Rev. Physiol. 9: 225, 1947) has stated that thiosulfate and creatinine may give higher clearances and therefore may be more suitable than inulin for measurement of glomerular filtration in normal man. However, inulin, being less likely to diffuse back into the blood stream, may give a better measure of glomerular filtration during partial renal ischemia.

Friedman and Byers have described a method for measuring glomerular filtration using allantoin (Proc. Soc. Exper. Biol. & Med. 66:522, 1947; Am. J. Physiol. 151:192, 1947).

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VIII. Measurement of Hepatic Blood Flow

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Inaccessibility of the liver and peculiarities of its vascular bed make measurement of hepatic blood flow particularly difficult. General anesthesia and traumatic surgical manipulation are necessary for direct determination of flow through the hepatic veins, hepatic artery and portal vein. Indirect methods based on clearance of various substances in the bile, similar to those used in measuring renal blood flow (9, 26), have not been developed because bile cannot be collected quantitatively in normal man or intact animals. However, the venous catheterization technique (7) permits determination of certain hepatic clearances and estimation of hepatic blood flow indirectly (3, 16, 18) despite this. Total volume of blood flowing out of the liver is derived from the hepatic artery (about 25 per cent) and the portal vein (about 75 per cent). Hence determination of any individual flow yields an incomplete picture of hepatic circulatory dynamics. Indeed, even with all three values at hand, the possibility of hepatic engorgement or disgorgement must be taken into account. Thus, the four variables—hepatic blood volume, arterial inflow,

portal inflow and venous outflow—are complexly interrelated. Methods for study of each are at best approximative and unsatisfactory. Simultaneous measurements of all four variables have not been made.

a) Hepatic volume.—Changes in volume of blood held in the liver may be estimated with fair accuracy on the basis of changes in liver volume. The influence of the cell mass cannot be ignored, but may be safely discounted when rapid enlargement or shrinkage of the liver occurs during circulatory adjustments. When large changes occur, as in congestive heart failure, manual palpation frequently provides an adequate qualitative measure. Physiologic alterations cannot be detected in this manner. Roentgenologic visualization is also of value in following massive shifts, although distortion of the image and difficulties of three-dimensional measurement limit accuracy. Thorotrast has been used to produce a denser liver shadow (20), but its dangerous radioactivity makes it unfit for use in study of normal human subjects. Methods based on its use have not been developed for study of animals.

Liver volume changes may be measured directly by the plethysmograph in animals. A waxed sheet-copper bell, notched above and below to prevent pressure on the inferior vena cava and portal vein, has been placed over the liver in cats, the opening of the bell directly facing the back of the animal and the tightly fitting abdominal wall furnishing an air-tight seal (13). Records of volume change in this device may be made from a metal tube set in its dome. Another method (17) employs a rigid collodion-gauze envelope around the left lobe of the liver in dogs. The plethysmograph is sutured in place at the "pedicle," and after a take-off tube is brought to the body surface the abdomen is closed. Records may be made after healing has occurred, without need for anesthesia or surgery. These methods yield data of questionable value, since the liver is placed under unphysiologic restraints, and extraneous factors other than liver volume may have an unpredictable influence.

b) Hepatic inflow.—Various mechanical devices have been used for measurement of blood flow through the hepatic artery and portal vein into the liver. Early in this century, Burton-Opitz (5) used the stromuhr in extensive studies of hepatic inflow. In the past 20 years the thermostromuhr (1, 14, 15, 21, 22) has been widely used (see p. 89). Many currently accepted concepts of hepatic hemodynamics are based on data obtained with it. Its use requires surgical isolation of the hepatic artery and portal vein so that the device may be placed around the vessels close to their entrance into the liver. However, recent work (11, 25) has cast doubt on the validity of thermostromuhr measurements under certain hemodynamic conditions, and the apparatus is falling into disuse. Other instruments, of which the rotameter (12, 23) appears to be the most satisfactory, have found little use (24) in study of hepatic circulation.

c) Hepatic outflow.—The thermostromuhr has also been used for measurement of the volume of blood flowing out of the liver. In one method (10) thermostromuhrs are placed on the thoracic inferior vena cava cra-

niad to the liver and on the abdominal vena cava between the orifices of the hepatic and renal veins. The difference between the volumes thus obtained is taken as the hepatic venous outflow. Or the inferior vena cava may be ligated just below the liver and a thermostromuhr affixed to the thoracic portion of the inferior vena cava for measurement of outflow (27). Both methods are subject to the criticisms that have already been noted.

Blalock and Mason (2) introduced a method which is of interest because it involves little surgical manipulation, no anesthesia and few ill effects. A blind brass cannula is passed, after local infiltration with novocain, into the right external jugular vein and thence through the superior vena cava and right atrium into the inferior vena cava. The cannula contains two tubes through which balloons of Penrose tubing affixed to the cannula may be inflated in the inferior vena cava above and below the entrance of the hepatic veins. Blood may then be removed from the isolated caval segment through perforations in the cannula between the balloons. The minute volume of blood thus obtained is equal to the hepatic outflow. The blood may be returned to the circulation by another vein. Experience is required for accurate placement of the cannula under fluoroscopic control. Care must be taken to avoid dead-space errors and collapse of the caval segment by negative pressure.

Direct methods such as these are wholly inapplicable in study of the human hepatic circulation. The technique of venous catheterization in man developed by Cournand and his co-workers (7, 8) has opened a new approach to study of liver function. It has proved possible to pass an extra-long ureteral catheter through the veins to the right atrium and thence into a hepatic vein in man for the purpose of sampling hepatic venous blood (3, 28). Only local anesthesia is required. There is no trauma or emotional disturbance. On the basis of this technique new methods are evolving for indirect measurement of hepatic blood flow, on the same principle that permits estimation of cardiac output by the Fick method and of renal blood flow by diodrast or sodium para-aminohippurate clearances. According to this principle, hepatic blood flow may be calculated by dividing the hepatic removal or formation rate of some substance, X, by the amount of X removed from or added to each milliliter of blood passing through the liver.

Bromsulfalein (BSP) has been used for this purpose because it is removed efficiently by the liver. It is administered by constant intravenous infusion at a rate sufficient to maintain plasma concentration between 1 and 2 mg per cent. Hepatic removal of BSP from the blood is considered equal to the rate of infusion when the plasma level is constant. When the concentration is changing, a correction may be made to account for the BSP retained or removed in excess of the infusion rate, assuming that, since BSP is bound to protein, it is distributed only in the plasma volume. This method of determining hepatic BSP removal is based on the belief that BSP is removed from the blood chiefly by the liver. Some extra-

hepatic removal may occur (6), but it is probably of little importance in normal subjects, since there is no significant extraction of the dye from blood perfusing other parts of the body. In addition, the important role of the liver is indicated by the striking reduction in BSP removal during liver disease. However, if there is extrahepatic removal, it is probably not disturbed by liver damage and, in these circumstances, it might contribute disproportionately to total removal, resulting in falsely high estimates of hepatic BSP removal and hepatic blood flow.

The amount of BSP removed from each milliliter of blood perfusing the liver is calculated as the difference between BSP concentrations in peripheral venous and hepatic venous blood. Here again, certain assumptions are necessary. First, the concentration of BSP in peripheral venous blood is considered equal to the concentration in blood entering the liver. BSP is present in the same concentration in peripheral venous and arterial blood, but part of the blood entering the liver is not arterial, having traversed the splenic and mesenteric vascular beds, where some of the dye may have been removed. In normal individuals this is immaterial, since nearly all portal blood passes through the liver before it returns to the heart, but when collateral circulation has developed, as in cirrhosis, a large portion of the portal blood escapes and the peripheral concentration of BSP can no longer be regarded as equivalent to the concentration in blood entering the liver. However, there is no evidence of BSP removal in the portal system outside the liver, and it seems likely that the assumption is still valid. Finally, mixed hepatic venous blood cannot be obtained because there are several separate hepatic veins. Hence a sample of blood from the right hepatic vein must be taken as representative of mixed hepatic venous blood. There are reasons for believing this usage valid, within reasonable limits. However, in consideration of the assumptions required, the value obtained by dividing the BSP removal rate by concentration difference between peripheral and hepatic venous blood is referred to as estimated hepatic blood flow (EHBF). This figure in human subjects (1440 ml/min/1.73m^2 average in 49 normal subjects (4)) agrees in general with data derived from experimental studies of animals and is supported by values obtained in man by the urea method (19).

Urea has been used in animals (16) and man (18) in the same manner as bromsulfalein. The rate of renal urea excretion may be taken as the rate of hepatic urea formation, the difference in hepatic and peripheral venous urea concentrations as the amount of urea added to each volume of blood passing through the liver and EHBF calculated as in the BSP method. The assumptions on which this procedure is based appear to be sound.

Development and improvement of indirect methods for quantitative measurement of hepatic blood volume, inflow and outflow are highly desirable. Better understanding of normal and abnormal hepatic hemodynamics should lead not only to more accurate and quantitative ap-

praisal of hepatocellular activity but to improved therapeutic techniques for dealing with disorders of the hepatic circulation.

Comment by Franz J. Ingelfinger

Dr. Bradley is to be congratulated on condensing this difficult subject into useful form. The reliability of the BSP method is still under discussion, but it appears as good as, if not better than, methods used in experimental animals. The following points might be stressed.

1. The method is sound, but BSP, although apparently the best available test substance, is not ideal, and development of the method may lie in identifying an even more satisfactory test substance.

2. Some investigators are using a high peripheral blood level of BSP in determining EHBF, but levels above 2 mg per cent may lead to errors. At high levels a small percentage amount, but a large absolute amount when considered in relation to over-all removal rate, may be removed extrahepatically. A considerable error may thereby be introduced when EHBF is calculated.

3. It is hard to give an absolute amount which expresses the renal excretion of BSP, since the absolute amount varies with the type of injection and the blood level. The renal loss of BSP can, however, be expressed as a percentage of BSP administered, whether given by injection or constant infusion.

Comment by Harold D. Green

I realize that reducing data to that expected for the standard 1.73 m^2 man has gained many adherents. However, I should prefer to see all data expressed per 1 m^2, as with basal metabolic rate. This simplifies the original calculation as well as computation of expected values for a person with a surface area differing from 1.73 m^2.

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IX. Quantitative Determination of Cerebral Blood Flow in Man

SEYMOUR S. KETY

Despite its obvious and fundamental importance, information on cerebral blood flow was not placed on a scientific basis until 1943, when Dumke and Schmidt (2) achieved the first quantitative measurements of cerebral blood flow under conditions approaching the normal. This was soon followed by studies of cerebral metabolism under similar circumstances (21). These measurements were made on species (rhesus or

spider monkeys) whose cerebral circulation resembles that of man in being readily isolated from the extracerebral blood supply of the head. A bubble flow meter (p. 80) interposed in the arterial supply to the brain yielded quantitative values for total cerebral blood flow.

In man so direct a procedure is out of the question, but various methods give indirectly some knowledge of the cerebral circulation in various clinical states. These include observation of the diameter and color of retinal vessels (1), measurement of cerebral arteriovenous oxygen differences (17), use of a heated thermocouple introduced into the internal jugular vein (6) and adaptation of the principle of the occlusion plethysmograph to the cranium (4). The limitations of these methods were discussed in an excellent symposium on cerebral circulation (25).

Two methods which appear to yield quantitative measurements have been developed: the nitrous oxide method (8, 10), based on the Fick principle; a dye dilution method (7), depending on the Stewart principle. These techniques are discussed here in some detail.

1. *Nitrous oxide method.*—The brain, unlike the kidney and liver, does not specifically and selectively remove foreign substances from the blood stream, hence the clearance techniques for measuring renal (p. 191) and hepatic (p. 199) blood flows are not applicable. It does, however, absorb by simple solution an inert gas which reaches it by way of the arterial blood, and this phenomenon forms the basis for measurement of cerebral blood flow by the Fick principle. This postulates, in its simplest form, that the quantity of any substance taken up in a given time by an organ from the blood which perfuses it equals the total amount of the substance carried to the organ by arterial inflow less the amount removed by venous drainage during the same time period. For the case of the brain uptake of N_2O, let

$Q_B]_u$ = quantity of N_2O taken up by the whole brain in time u measured from the start of inhalation,
$Q_A]_u$ = quantity brought to the brain by arterial blood in time u,
$Q_V]_u$ = quantity carried away by cerebral venous blood in time u,
A = arterial N_2O concentration,
V = venous N_2O concentration,
TF = total cerebral blood flow/min,
CBF = cerebral blood flow per unit weight of brain/min,
W = brain weight.

From the Fick principle:

$$Q_B]_u = Q_A]_u - Q_V]_u,$$

but since both A and V are variables with respect to time (Fig. 1),

$$Q_A]_u = TF \int_0^u A\,dt$$

and

$$Q_V]_u = TF \int_0^u V\,dt,$$

whence

$$Q_B]_u = TF \int_0^u (A - V)\,dt$$

or

$$TF = \frac{Q_B]_u}{\int_0^u (A - V)dt} \tag{1}^*$$

or, in terms of unit weight of brain,

$$CBF = \frac{Q_B]_u/W}{\int_0^u (A - V)dt}. \tag{2}$$

The quantity $\int_0^u (A-V)dt$ is readily obtained from the respective arterial and cerebral venous curves. The numerator, or cerebral concentration of nitrous oxide, is not obtainable directly in man. If the time u is sufficiently long, however, equilibrium will have occurred between brain and blood leaving the brain with respect to nitrous oxide tension. At that time,

$$\frac{Q_B]_u}{W} = V_u S, \tag{3}$$

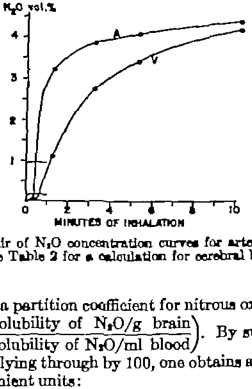

FIG. 1.—Typical pair of N_2O concentration curves for arterial (A) and internal jugular (V) blood. See Table 2 for a calculation for cerebral blood flow from these curves.

where S represents a partition coefficient for nitrous oxide between brain and blood $\left(S = \frac{\text{solubility of } N_2O/\text{g brain}}{\text{solubility of } N_2O/\text{ml blood}}\right)$. By substituting appropriately and multiplying through by 100, one obtains a value for cerebral blood flow in convenient units:

$$CBF = \frac{100\, V_u S}{\int_0^u (A - V)dt}, \tag{4}$$

where CBF is expressed as ml of blood flow/100 g of brain/min.

* This is a general formula for the Fick principle applying to any organ or the whole body and to any substance. In its special use for determination of cardiac output with O_2 or CO_2 the denominator becomes simply $(A - V)t$ since the arteriovenous differences of these gases are presumed to be constant.

Application of this formula to practical measurement of cerebral blood flow, although theoretically valid, necessitates certain assumptions. These were all subjected to experimental evaluation with the following conclusions: (1) Blood from one internal jugular vein at the level of the superior bulb represents mixed cerebral venous blood (10) with only slight contamination by blood of extracerebral origin (21). (2) After 10 min of inhalation of a constant tension of nitrous oxide the venous blood is in equilibrium with the brain with respect to nitrous oxide tension (12). Therefore the value of u in equation (4) may be taken as 10 min. (3) The partition coefficient of nitrous oxide between brain and blood (factor S in equation (4)) equals unity (12). In addition, simultaneous measurement of cerebral blood flow in monkeys by the nitrous oxide method and the bubble flow meter showed excellent agreement (10).

PROCEDURE

The N_2O concentration curves are drawn on the basis of five pairs of blood samples from the superior bulb of the internal jugular and an artery taken at intervals throughout a 10 min period of inhalation of 15 per cent nitrous oxide. From these curves the cerebral blood flow is calculated by means of equation (4).

a) *Blood sampling.*—For taking the accurately timed serial blood samples from artery and internal jugular vein, manifolds of 3-way stopcocks† (Fig. 2) are convenient. The sampling syringes (10 ml Luer-Lok) are prepared beforehand by lightly oiling the plunger with paraffin oil, filling the dead space with heparin solution and sealing with the closed hubs of discarded hypodermic needles. Transparent plastic tubing of small bore‡ connects the manifold to the needle (19 gauge, 3 in. spinal) through suitable adaptors.§ The needle is placed in the superior bulb of the internal jugular after procaine infiltration according to the technique of Myerson, Halloran and Hirsch (18) modified by Gibbs, Lennox and Gibbs (5). The superior bulb of the internal jugular lies fairly constantly in a line between the anterior tip of the mastoid process and the posterior edge of the external auditory meatus and 3–4 cm below the surface. The needle point is inserted about 1 cm below the mastoid process and directed upward in the line of the internal jugular at an angle of 45–60°. The sudden release of resistance as the needle enters the vein is similar to that which occurs in a spinal tap. If bone is encountered the angle is too sharp, and the needle is almost completely withdrawn and an angle closer to the perpendicular chosen. This technique is readily tolerated and very dependable; incidence of failure can be less than 1 per cent. For arterial blood, femoral or brachial arteries are used. Once in place, the needle is carefully connected to the plastic tubing filled with sterile heparin

† *Constructed by Mr. D. W. T. Cochrane, Department of Pharmacology*, University of Pennsylvania.

‡ Transflex, 14 gauge, made by Irvington Insulator Co., Irvington, N. J.

§ The manifolds are sterilized by autoclaving, but the plastic tubing is best sterilised in 70 per cent alcohol.

solution (10 mg/ml) by means of the end syringe containing 3 ml of heparin (Fig. 2). Just before each sample is taken 4 ml of blood is drawn into this syringe to clear the system, and at the conclusion of each sample 4 ml of the blood-heparin mixture in this syringe is pushed back to prevent clotting.

The following samples of 6–8 ml each are taken: X, a blank taken from the vein just before beginning of N_2O inhalation; 1A and 1V, taken at a constant rate (1 ml every 10 sec) from onset of N_2O inhalation over the first minute; 2A and 2V, from 1 min 5 sec to 1 min 30 sec; 3A and 3V, from 2 min 45 sec to 3 min 15 sec 4A and 4V, from 4 min 45 sec to 5

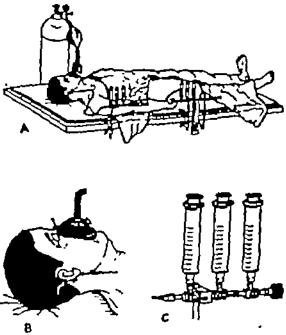

FIG. 2.—*A*, apparatus used in measurement of cerebral blood flow by nitrous oxide method, showing gas system, manifolds attached to needles in internal jugular and femoral artery and arterial pressure manometer attached to its manifold. *B*, detail of portion of the needle in the superior bulb of the internal jugular. *C*, detail of a manifold showing 3-way stopcocks, to which are attached three sampling syringes; also, part of the end syringe containing heparin and blood and used for flushing before and after sampling. (Drawing by Dr. E. L. Foltz.)

min 15 sec; 5A and 5V, from 9 min 45 sec to 10 min 15 sec. The actual times are inconsequential so long as they are accurately noted and fairly evenly spaced. As soon as possible after each sample is taken the syringe is removed from the manifold and any small bubble of air expelled; it is then sealed and placed in cracked ice until analysis. Although it is possible to analyze any of these samples for CO_2 and O_2, it is preferable to take separate samples for this, one pair at the time of the X sample, and again after the 5 min N_2O sample.‖ The N_2O mixture consists of 15 per cent N_2O, 21 per cent O_2, 64 per cent N_2 obtained from a medicinal

‖ By the mathematical nature of the problem, cerebral blood flow is largely determined at the end of 5 min.

gas company and kept in large cylinders under pressure. Below 2000 lb/in.^{2} pressure all constituents are gaseous and the mixture is homogeneous.

The gas is administered in an open system through an anesthesia bag and tightly fitting mask equipped with inspiratory and expiratory valves. It is important that these valves be competent and the mask fit perfectly. The mask may be fastened to the face by a suitable halter or rubber cement or, best of all, held snugly on the face for the required 10 min by an assistant. Flow of gas should be considerably in excess of the subject's requirements as a further precaution against leakage of room air into the mask. Unless the patient breathes a constant tension of N_2O throughout the flow the arterial curve will not be a smooth function and cannot accurately be drawn from only five samples. A rebreathing system is not desirable since the high solubility of N_2O will cause the arterial curve to fall off fairly rapidly. In our experience 15 per cent N_2O causes no appreciable physiologic or mental effects.

A second measurement can be made on the same subject while the needles are still in place. At least 20 min must elapse between determinations to permit cerebral N_2O desaturation; of course, a blank blood sample is taken just before the second determination.

b) Analysis of blood samples for N_2O.—Because of the number of analyses necessary for each blood flow determination, the method of Orcutt and Waters (19) has been modified to make it simpler, more accurate and more rapid.

Reagents: caprylic alcohol, in dropper bottle. Oxygen absorber mixture—10 parts of powdered sodium hydrosulfite ($Na_2S_2O_4$) and 1 part of sodium anthraquinone beta-sulfonate. 1N potassium hydroxide. De-aerated CO_2–O_2 absorber.

Measure 11 g of the powdered oxygen absorber mixture into a 100 ml beaker by means of a marked test tube, add 50 ml of normal potassium hydroxide and stir for 30 sec with minimal agitation of the surface. Then quickly filter through a small pad of absorbent cotton into a 50 ml Erlenmeyer flask and immediately transfer to the chamber of a Van Slyke-Neill manometric apparatus. Enough reagent will have been lost in these processes that the solution will not completely fill the 50 ml chamber. Seal the upper stopcock with mercury and subject the reagent to evacuation by bringing the mercury level in the chamber a few centimeters below the 50 ml mark. Add 1 or 2 drops of caprylic alcohol to the chamber before adding the reagent to prevent foaming. Shake the chamber 3 min, then permit the reagent to rise and eject the bubble of nitrogen. Repeat de-aeration until no more gas is evolved. This rarely requires more than two shakings. Then run 1 ml of the reagent into the cup from the chamber to act as a seal and transfer the rest of the reagent to a 50 ml glass syringe (lubricated with oil and equipped with a rugged capillary tip 8 cm long) with the aid of a rubber tip temporarily attached to the tip of the capillary and pressed firmly against the bottom of the

solution (10 mg/ml) by means of the end syringe containing 3 ml of heparin (Fig. 2). Just before each sample is taken 4 ml of blood is drawn into this syringe to clear the system, and at the conclusion of each sample 4 ml of the blood-heparin mixture in this syringe is pushed back to prevent clotting.

The following samples of 6–8 ml each are taken: X, a blank taken from the vein just before beginning of N_2O inhalation; 1A and 1V, taken at a constant rate (1 ml every 10 sec) from onset of N_2O inhalation over the first minute; 2A and 2V, from 1 min 5 sec to 1 min 30 sec; 3A and 3V, from 2 min 45 sec to 3 min 15 sec 4A and 4V, from 4 min 45 sec to 5

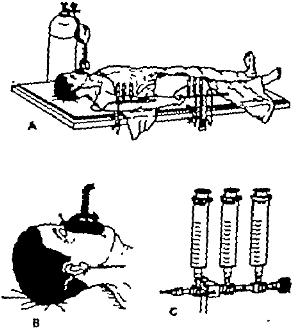

FIG. 2.—*A*, apparatus used in measurement of cerebral blood flow by nitrous oxide method, showing gas system, manifolds attached to needles in internal jugular and femoral artery and arterial pressure manometer attached to its manifold. *B*, detail of portion of the needle in the superior bulb of the internal jugular. *C*, detail of a manifold showing 3-way stopcocks, to which are attached three sampling syringes; also, part of the end syringe containing heparin and blood and used for flushing before and after sampling. (Drawing by Dr. E. L. Folts.)

min 15 sec; 5A and 5V, from 9 min 45 sec to 10 min 15 sec. The actual times are inconsequential so long as they are accurately noted and fairly evenly spaced. As soon as possible after each sample is taken the syringe is removed from the manifold and any small bubble of air expelled; it is then sealed and placed in cracked ice until analysis. *Although it is* possible to analyze any of these samples for CO_2 and O_2, it is preferable to take separate samples for this, one pair at the time of the X sample, and again after the 5 min N_2O sample.‖ The N_2O mixture consists of 15 per cent N_2O, 21 per cent O_2, 64 per cent N_2 obtained from a medicinal

‖ By the mathematical nature of the problem, cerebral blood flow is largely determined at the end of 5 min.

where $X = f_{N_2O}[r_s - (r_r + C_w)]$, f_{N_2O} being a manometric factor corresponding to the temperature of analysis.

It is much more convenient not to denitrogenate the subject beforehand but to keep the total inert gas tension in the mixture equal to that in room air. This necessitates correction for nitrogen** which can be derived from knowledge that in such circumstances the total inert gas tension in the blood would be kept constant and that the manometer reading on the Van Slyke apparatus is the sum of the readings due to nitrogen and nitrous oxide. From Henry's law, the general gas equation and the known constants for nitrogen and nitrous oxide the foregoing formulae are found to apply, but a different set of factors (f'_{N_2O}) must

TABLE 1.—MANOMETRIC FACTORS FOR CALCULATION OF BLOOD N_2O IN VOL. % FROM 2 ML SAMPLE OF BLOOD

Temp. Analysis, C	f_{N_2O}	f'_{N_2O}	ΔP_w
20	0.1400	0.1456	1.1
21	.1393	.1449	1.2
22	.1386	.1441	1.2
23	.1379	.1434	1.3
24	.1372	.1427	1.4
25	.1365	.1420	1.4
26	.1358	.1412	1.5
27	.1351	.1405	1.6
28	.1344	.1398	1.7
29	.1337	.1390	1.7
30	.1330	.1383	1.8
31	.1323	.1376	1.8
32	0.1316	0.1368	1.9

NOTES.—(1) Use f'_{N_2O} when gas mixture consists of 21% O_2, 64% N_2, 15% N_2O and subject has been breathing air previously. (2) Use f_{N_2O} when gas mixture consists wholly of O_2 and N_2O and subject has been denitrogenated previously. (3) $C_w = (t_a - t_b)\Delta P_w$. If temperature difference between analysis and blank is several degrees, use that ΔP_w which corresponds to the mean temperature.

Retain the algebraic sign of C_w in substituting in the formula:

$$\text{vol. \% } N_2O = f_{N_2O} \text{ or } f'_{N_2O}\,[r_s - (r_r + C_w)] - X$$
$$X = f_{N_2O} \text{ or } f'_{N_2O}\,[r_s - (r_r + C_w)]$$

be used. In those conditions the value for X will be close to 1.15 volume per cent unless a previous flow measurement has been performed. If 20 min elapses between successive flows, nitrous oxide desaturation will be practically complete and X will be only a few tenths of a volume per cent over 1.15. Table 1 gives values for f'_{N_2O} and ΔP_w for temperatures from 20 to 32 C. With good care duplicate analyses should agree within 0.05 volume per cent. After sufficient skill has been acquired it is possible to forego duplicate analyses, using the smoothness of the resultant curves as a check on individual analyses.

In the presence of N_2O the usual analysis for O_2 and CO_2 must be modified to correct for the solubility of N_2O in absorbing reagents; i.e.,

** Since solubility of N_2O in blood is over 32 times that of N_2, the error due to nitrogen, even if uncorrected, is small; with proper correction it vanishes.

cup. Keep the reagent in the capillary out of contact with air except for negligible diffusion up the capillary. It may be kept overnight in a refrigerator by sealing the tip.

In making an analysis add 2 drops of caprylic alcohol to the chamber of the manometric apparatus followed by 9 ml of distilled water. De-aerate the mixture for 2 min. Thoroughly shake the blood sample in its syringe by means of a small amount of mercury drawn up into it just before analysis. Place a small drop of caprylic alcohol in the tip of a 2 ml Ostwald-Van Slyke pipet equipped with stopcock, and by means of a short rubber tube fill the pipet smoothly to the mark with blood from the syringe. Allow the de-aerated distilled water to rise to the 5 ml mark in the cup, add 2 ml of blood to the chamber and wash in with 1 ml of de-aerated water. (It is important that no air bubbles be admitted to the chamber during these procedures; if one is present at this point it must be expelled.) Remove the rest of the water in the cup by gentle suction. Immediately add 3 ml of the de-aerated CO_2–O_2 absorber to the cup and then run the lower 2 ml into the chamber. Seal the chamber with *mercury and extract the mixture with the mercury level at 50 ml for 3* min. Then allow it to rise smoothly to the 2 ml mark of the buret and make readings of pressure (r_a) to 0.1 mm and temperature (t_a) to 0.1°. Expel the mixture from the chamber and begin the next analysis without washing the chamber. Thus in this simplified method oxygen and carbon dioxide are absorbed immediately and never extracted from the blood. The only gases present in significant quantity which are extracted and measured are nitrous oxide and nitrogen.

Just before the blood analyses are started a blank determination is made, *using 2 ml of de-aerated water instead of blood (run the de-*aerated water up to the 5 ml mark, then down to the 2 ml mark on the cup, add O_2 absorber, extract, read pressure (r_c) at 2 ml and temperature (t_c)). This blank reading, corrected for changes in water vapor pressure resulting from any temperature change between the blank and each analysis (C_w), is used as the quantity to be subtracted from the readings on the blood analyses. It is important, therefore, since a calculated r_c is used for each analysis, that more than usual care be taken to insure accuracy in the volumes of reagents added to the chamber. In addition to *the water blank, a blood blank value (X) must be obtained by analysis of* the X sample obtained just before inhalation of nitrous oxide. This is part of the correction for nitrogen and other inert gases and for any nitrous oxide remaining in the subject's blood from a previous period of inhalation.

If the subject were almost denitrogenated beforehand and the inhaled mixture consisted only of N_2O and O_2, blood N_2O content would be calculated as follows:

$$\text{vol. \% } N_2O = f_{N_2O}[r_a - (r_c + C_w)] - X,$$

TABLE 3.—BLOOD FLOW AND OXYGEN CONSUMPTION OF HUMAN BRAIN (85 OBSERVATIONS ON 15 HEALTHY YOUNG MEN)

	MEAN	σ*
Cerebral blood flow (ml/100 g/min)	54	±12.6
Cerebral O_2 consumption (cc/100 g/min)	3.3	± 0.4
Cerebrovascular resistance (mm Hg/ml blood/100 g brain/min)	1.6	± 0.4
Cerebral a-v O_2 diff. (vol. %)	6.3	± 1.2
Cerebral respiratory quotient	0.99	± 0.09
Mean femoral arterial BP (mm Hg)	86	± 6.8

* Among the 15 subjects.

value of the function in column (7) at 10 min represents cerebral blood flow expressed as ml/100 g of brain/min.

With the value for cerebral blood flow it is now possible to arrive at a measurement of some extremely important functions. Utilization or production by the brain of any substance capable of accurate analysis in arterial and cerebral venous blood is estimated quantitatively by substitution in the transposed Fick formula. Thus, for cerebral utilization of oxygen (CMR_{O_2}),

$$CMR_{O_2}\ (\text{cc } O_2/100 \text{ g brain/min}) = CBF \times \frac{(A - V)_{O_2}}{100}$$

if $(A - V)_{O_2}$ is expressed as volumes per cent. A value for cerebrovascular resistance (CVR) is calculable from mean carotid blood pressure, internal jugular pressure and cerebral blood flow:

$$CVR = \frac{\text{mean carotid BP} - \text{mean jugular BP (mm Hg)}}{CBF\ (\text{ml}/100\ \text{g/min})}.$$

CVR is obtained in convenient units representing the pressure necessary

TABLE 4.—CEREBRAL BLOOD FLOW AND O_2 CONSUMPTION DETERMINED BY NITROUS OXIDE METHOD

CONDITION	NO. OF OBSERVATIONS	CBF ml/100 g/min	CMR_{O_2} cc/100 g/min
Normal			
Resting	35	54	3.3
Hyperventilation	13	34	3.7
5–7% CO_2	8	93	3.5
85–100% O_2	6	45	3.2
10% O_2	7	73	3.3
Pentothal anesthesia	1	52	1.9
Hypertension	10	54	3.4
Increased intracranial pressure	11	43	2.9
Cerebral hemangioma	2	164	3.3
Schizophrenia			
Resting	30	54	3.3
Pentothal seminarcosis	8	53	3.3
Postelectroshock	7	37	3.1
Insulin hypoglycemia	5	61	2.6
Insulin coma	5	63	1.9
Diabetic acidosis	8	45	2.7
Diabetic coma	6	65	1.7
Epilepsy	12	51	3.4

extract at 50 ml for 2 min after adding each reagent, then bring to 2 ml and read.

c) Calculation of cerebral blood flow (CBF).—When the nitrous oxide analyses are completed the values are plotted against time. The time of each sample is taken as the midtime of the interval over which the sample was taken except for the first pair, taken at a constant rate over the first minute (and therefore already integrated) which are plotted as straight lines (Fig. 1). Smooth curves are then drawn through the arterial and venous points and so constructed over the first minute that the average samples obtained (1A and 1V) approximate the respective integrals of the curves. From these smooth curves the integral of the arteriovenous difference can be obtained over the 10 min period by means of the trapezoid rule. This yields the denominator in the equation

TABLE 2.—Typical Calculation for CBF Using Data of Figure 1

(1) t	(2) A_{N_2O}	(3) V_{N_2O}	(4) $(A-V)_{N_2O}$	(5) $\int_{t-1}^{t}(A-V)dt$	(6) $\int_{0}^{t}(A-V)dt$	(7) $\frac{100\,V_t}{\int_0^t (A-V)dt}$
1	2.98	0.77	2.21	0.80	0.80	96.2
2	3.58	1.86	1.72	1.97	2.77	67.2
3	3.81	2.58	1.23	1.47	4.24	60.9
4	3.93	3.06	0.87	1.05	5.29	57.8
5	4.03	3.36	.67	0.77	6.06	55.4
6	4.12	3.60	.52	.60	6.66	54.0
7	4.20	3.78	.42	.47	7.13	53.0
8	4.27	3.94	.33	.37	7.50	52.6
9	4.33	4.08	.25	.29	7.79	52.4
10	4.39	4.19	0.20	0.23	8.02	52.2

CBF = 52 ml/100 g/min.

Notes.—Column (1), time from onset of inhalation of N_2O mixture. Columns (2) and (3), values read from smooth curves. Column (4), arteriovenous N_2O difference. Column (5), average of $(A-V)_t$ and $(A-V)_{t-1}$ except for the first minute, which is obtained directly as the difference between the first pair of samples taken at a constant rate throughout the first minute (1A = 0.95, 1V = 0.15 vol. %). Column (6), sum of column (5) up to and including the particular value of t. CBF is simply the value in column 7 at t = 10 min.

for cerebral blood flow, the numerator being venous concentration at 10 min. In practice it is preferable to build this calculation up in a series of steps of 1 min each from the beginning to the end of 10 min. An example of a typical complete calculation is given in Table 2. Here the function in column (7) decreases smoothly over the first 5 or 6 min but, as 10 min is reached, tends to level off. This tendency serves as an internal check on the final result since equilibration between brain and venous blood should be complete within 10 min. In most cases this function is not perfectly constant at that time, indicating the small amount of contamination from extracerebral sources. Rapid fall of this function even at 10 min is evidence that contamination is significant and the study should be discarded. This was observed only twice in 100 studies. Since the partition coefficient (S) is unity and equilibration time (u) 10 min, the

heparin solution between samplings. The dye (0.2 per cent solution of Evans blue) is injected at a constant rate (1 ml/min) through a 22 gauge needle in the internal carotid. Injection rate is automatically maintained by a constant speed injector. After at least 2 min is allowed for equilibrium to be established, a sample of blood is taken from the venous catheter and discarded, followed by simultaneous samples of 10 ml each from the internal jugular and from a needle in the femoral artery. These two samples are centrifuged and the dye concentration in the plasma is determined by means of photoelectric colorimetry. The concentration of dye in the whole blood is equal to its concentration in plasma multiplied by the plasma hematocrit. Although it is possible to correct for hemolysis (7), this should not occur if lightly oiled syringes are used for collection of blood samples.

Although the Stewart principle permits rapid determinations, much of that advantage is lost in this particular application by the 2 min equilibration period. A proposed modification of the foregoing method gives determinations of equal validity but requires a small fraction of the time (21). In this, equation (1) is used. An accurately measured quantity of the dye (1 ml of a 0.2 per cent solution) is injected into the internal carotid with a calibrated tuberculin syringe, the injection requiring only 1 or 2 sec. From the beginning of injection and for an accurately measured interval of 20 sec, blood samples are taken at a constant rate (0.5 ml/sec) from the internal jugular and a peripheral artery. This interval is sufficient for all the dye to be washed through the cerebral circulation. The samples automatically integrate the dye concentrations, so that equation (1) becomes:

$$F = \frac{I}{(V - A)t},$$

where I is the quantity of dye injected (mg); V and A, whole blood concentrations of dye (mg/ml), and t, time over which the samples were taken (min).

The Stewart principle is only imperfectly applicable to the cerebral circulation since dye injected into only one of the four major sources of cerebral blood (unlike nitrous oxide, which enters the brain via all the arteries) does not mix uniformly with the entire cerebral circulation (21). This introduces a major source of error into the dye injection methods which could possibly be overcome by injecting the dye into both internal carotids or by taking samples from both internal jugulars or from one internal jugular with the other temporarily occluded. These possibilities remain to be explored.

NOTE.—This section was reviewed by Eugene M. Landis.

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to force 1 ml of blood/min through 100 g of brain. This may be converted to absolute units by an appropriate factor.

d) Typical values obtained.—Table 3 shows mean results and standard deviations of 35 observations on 15 healthy young men (10). Table 4 gives mean values obtained in various clinical conditions (9, 11, 13–16, 22, 23). Application of this method is not confined to the brain, for it can be used in any organ where representative and relatively uncontaminated venous blood can be obtained, where the time of organ:venous blood equilibration (u) is conveniently short and where a partition coefficient (S) can be determined. Application of the method to study of the coronary circulation is under way (3). In its application to the brain the experimental error of this method is fairly small. For CMR_{O_2} the standard deviation of the differences between duplicates taken 30 min apart is $\pm$ 0.19 cc O_2/100 g/min (10).

2. *Dye dilution method.*—The Stewart principle (24) on which this method is based may be derived as follows: Consider a substance (Evans blue dye), which does not leave the circulation in question, to be injected into an artery going only to that organ and to suffer such mixing in the organ that its concentration is uniform in all of the veins which leave it. Let

$q_i]_{t_1}^{t_2}$ = quantity of dye which enters the organ in a time interval $(t_2 - t_1)$,

$q_o]_{t_1}^{t_2}$ = quantity which leaves in time $(t_2 - t_1)$.

Assuming a steady state,

$$q_i]_{t_1}^{t_2} = q_o]_{t_1}^{t_2}.$$

Let

A = concentration of dye in arterial blood by virtue of recirculation,
V = concentration of dye in venous blood from the organ,
I = quantity of dye injected in time $(t_2 - t_1)$.

$$q_i]_{t_1}^{t_2} = I + F \int_{t_1}^{t_2} A\,dt, \text{ and } q_o]_{t_1}^{t_2} = F \int_{t_1}^{t_2} V\,dt,$$

where F is total flow through the organ, whence

$$F = \frac{I}{\int_{t_1}^{t_2} (V - A)dt}. \tag{1}$$

Gibbs, Maxwell and Gibbs (7), in their application of this principle to the brain, use a formula which is equation (1) in differential form:

$$F = \frac{R}{V - A}, \tag{2}$$

where R is the constant rate of dye injection (mg/min). According to their method, the bifurcation of one carotid and the internal jugular is exposed under local anesthesia and a catheter introduced through a branch of the internal jugular up to the superior bulb. This is filled with

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X. Miscellaneous Methods

HAROLD D. GREEN

A number of clinical methods for study of peripheral vascular diseases were reviewed by Allen, Barker and Hines (1). Therefore they are not discussed here. Those which may be of value for investigative purposes include: the *time for reactive hyperemia* to develop after a period of ischemia; the *color and temperature of the skin* before and after ischemia; *arteriography and venography by injection* of radiopaque substances in the living subject; *oscillometry*, which both Allen and his co-workers and the editor feel has little practical value; *circulation time*, which is fully covered by these authors, including tables of normal values; *histamine wheal and flare test; saline wheal test; measurements of oxygen content of venous blood* and of *arteriovenous oxygen difference*.

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through the cognate bed as a result of changes in pressure of a few millimeters of mercury in the outflow system or in the pressure in collateral venous channels.

Studies of the relationship of perfusion pressure to flow through the cognate bed in measurement of peripheral resistance may be satisfactorily accomplished in most vascular regions by perfusing the collateral arteries at the same pressure as that of the cognate artery. However, technical errors are very likely whenever the flow into the collateral arteries is extremely large or the anastomotic communications are prominent.

In flow studies in the distal part of extremities the exchange of blood

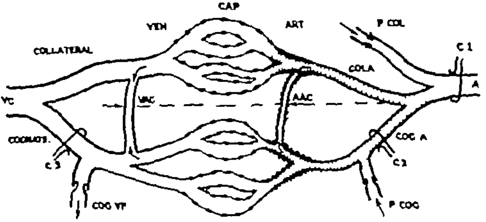

FIG. 1.—Anatomic arrangement of peripheral circulation. Upper half, collateral vascular bed; lower half, cognate vascular bed. *A*, aorta; $C_{1,2,3}$, clamps for compressing various vessels; *P COL*, *P COG*, points at which arterial inflow and pressure are measured in collateral and cognate systems; *COL A*, *COG A*, collateral and cognate supply arteries; *ART*, arteries and arterioles; *CAP*, capillaries; *VEN*, venules and veins; *AAC*, arterial anastomotic channel; *VAC*, venous anastomotic channel; *COG VF*, point at which cognate venous outflow is measured; *VC*, vena cava; arrows, points at which tourniquets presumably interrupt flow between collateral and cognate vascular beds. (Reprinted from Am. J. Physiol.)

across both venous and arterial anastomotic channels may be effectively prevented by application of wire ligatures which tightly compress all structures except the cognate artery and vein and the nerves (1).

II. Measurement of Effective Collateral Circulation

Effective collateral flow is defined as the rate at which blood will flow from the collateral beds through the anastomotic channels and thence through the cognate bed immediately after occlusion of the cognate artery. Measurement of backflow from the distal end of the cognate artery provides a rough measure of effective collateral flow. A better estimate of effective collateral flow with respect to normal flow through the cognate bed at mean aortic pressure is given by the ratio of the pe-

COLLATERAL CIRCULATION

HAROLD D. GREEN

I. Artifacts in Measurement of Flow

PERIPHERAL RESISTANCE changes and vasomotor reactions are frequently studied by measuring the blood flow through specific vascular beds by recording either the flow into the artery supplying the region or the flow out of the vein draining it. However, either the inflow or the outflow may differ considerably from the flow through the vascular bed whenever the pressure in the cognate artery or vein differs from that in the corresponding collateral vessels. This discrepancy is due to the variable exchange of blood across the arterial and venous anastomotic channels between the vascular bed being studied and the collateral vascular beds.

The anatomic arrangement of the peripheral circulation is shown in Figure 1. The lower half of the figure represents the vessels in which vasomotor reactions are being studied—the "cognate" system. The upper half represents all adjacent vessels having arterial and/or venous anastomotic communications with the cognate capillary bed—the "collateral" systems.

The magnitude of exchange of blood across the arterial anastomotic channels may be studied by comparing the flow of blood from a reservoir into the cognate artery at a given perfusion pressure while the collateral arteries are receiving blood (*a*) at higher, (*b*) at the same and (*c*) at lower pressures. In experiments on the hindextremity of the dog, in which the collateral arteries were receiving blood from the aorta and resistance to flow in the arterial anastomotic channels was small in relation to that in the cognate capillary bed, it was observed that: (*a*) flow into the cognate artery at a perfusion pressure 15 mm Hg greater or less than aortic mean pressure differed from the flow through the cognate bed by 40–300 per cent, and (*b*) although flow through the cognate bed was unchanged, flow into the cognate artery under a constant head of pressure provided by a reservoir increased 40–1000 per cent as a result of fall in pressure in the collateral arteries such as might be produced by a 50 per cent decline in aortic pressure.

In the presence of prominent venous anastomotic channels, outflow from the cognate vein may vary from 25 to 200 per cent of the flow

CARDIAC OUTPUT AND CONTRACTILITY

HAROLD D. GREEN

I. Cardiac Contractility

THIS WAS MEASURED DIRECTLY by Walton and Brodie (36), who recorded the force just necessary to prevent shortening of a given group of myocardial fibers as the heart responded to various agents presumed capable of modifying the inherent cardiac contractility. (See also discussion, p. 247.) Warren, Weens and James (abst., Am. Soc. Clin. Investigation, 1948) have described a cinematographic technique for studying heart action which may be useful in evaluating contractility.

II. Physical Methods for Cardiac Output

Measurement of cardiac output by a physical method which would permit continuous quantitative and relatively instantaneous determinations is a highly desirable, but incompletely attained, objective. Means which have been studied include: the pneumotachygram (3, 7, 9, 16, 17, 20), the impedance cardiogram (1, 2, 6, 23, 24, 29, 30), pulse wave velocity and contour (13, 18, 26, 27, 37) and the ballistocardiograph (5, 15, 21, 22, 25, 31, 32). The editor feels that the first two have not demonstrated their adequacy as research tools. Because of limitations of space, discussion of these four methods is omitted. The references cited and, in particular, reviews by Hamilton (11, 12) should be consulted for details.

III. Injection Methods

Stewart (34, 35) first attempted the computation of cardiac output from the arteriovenous difference in concentration of a substance (sodium chloride or a dye) injected at a constant rate into a vein. The method, revived by Wiggers (38), has been reviewed by Hamilton (11, 12). Recent studies suggest that the dye injection method may give results with reliability comparable to that of the direct Fick method (8, 14). With these techniques analysis is simpler than that for oxygen, and

ripheral arterial pressure in the cognate artery minus 20 to the mean aortic pressure. A more accurate measure of effective collateral flow is obtained by recording flow into the cognate artery while perfusing the cognate and collateral arteries at a pressure equal to the peripheral arterial pressure in the cognate artery (above) and comparing this with the flow through the cognate bed at mean aortic pressure. In various portions of the hind-extremity of the dog the effective collateral flow varied from 9 to 85 per cent of normal flow through the cognate bed (for details see (1)).

NOTE.—Sections I and II were reviewed by Donald E. Gregg.

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cardiac catheterization or puncture is not necessary. White (37a) has described a modification of the saline injection method for cardiac output in which the conductivity of femoral artery blood is recorded continuously with a cathode ray oscillograph.

IV. Gasometric Methods

Various gasometric methods have been devised (10). They principally involve the use of the Fick O_2 difference concept, but involve elaborate techniques for attempting to determine the mixed venous O_2 content without having recourse to cardiac catheterization or puncture. They give lower readings in general than the direct Fick procedure, which the editor prefers (4, 19, 28). (See p. 224.)

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V. Determination of Cardiac Output in Man by Right Heart Catheterization

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The Fick principle has been the most widely accepted basis for determination of cardiac output in the intact experimental animal. Application of the direct Fick principle to measurements in man was hampered by difficulty in obtaining samples of mixed venous blood until the report by Forssmann (5) in 1929 that a catheter could be passed through the venous system into the human heart and later demonstration of its safety and utility by Cournand and his colleagues (2, 3) led to widespread use in man (3, 8, 11, 12). Several thousand catheterizations have been performed in various laboratories with no record of serious untoward effect. However, it is a potentially dangerous procedure and should only be carried out with considerable care and thoughtfulness.

The Fick principle can be applied in various ways, but in determination of cardiac output by the catheter technique, measurement of oxygen values has proved to be most useful. In brief, the principle is

$$\text{cardiac output (l/min)} = \frac{O_2 \text{ consumption (cc/min)}}{\text{a-v } O_2 \text{ diff. (cc/l)}}.$$

Samples of arterial blood are obtained by direct puncture and those of mixed venous blood by catheterization of the right heart. From these data a-v oxygen difference can be calculated. Oxygen consumption is measured either by a standard metabolism technique or by an open circuit method with collection and analysis of samples of expired air.

EQUIPMENT

a) *X-ray apparatus.*—Although x-ray equipment is highly desirable, it is not essential. The procedure is usually carried out with the subject horizontal on a fluoroscopic table that is covered with a mattress or pad so that the patient may remain on it for some time without discomfort. The usual technique for fluoroscopy of the chest is followed. Protection against excessive radiation of the patient and exposure of the operators is required. A timing device is useful to warn if fluoroscopy is unduly prolonged. The operator and his assistants should wear leaded aprons. Since it is practically impossible for the operator to wear leaded gloves, extreme care should be taken to avoid placing the hands in the direct beam of the x-ray. At times it is desirable to have facilities available for taking x-ray films, but this is not required.

b) *Catheters.*—The catheters used are only slightly different from those commonly used in ureteral catheterization.* They are 100 cm long and made of woven fabric with a plastic covering. A small angulation a few centimeters from the tip permits direction of its progress by rotation of the external end. The other end is fitted to an adaptor similar to the hub of a hypodermic needle. These catheters can be sterilized by autoclaving as for rubber goods.

c) *Other instruments.*—A small set of sterile instruments is needed for skin incision and isolation of the vein, and a second set is used for closing the incision after the procedure is completed. The following items are contained in the kits used at Emory University:

Instrument Kit	Sewing Kit
Tourniquet	Needle holder
3-way stopcock with rubber tubing	Small scissors
1 scissors	2 curved suture needles, threaded
1 or 2 towel clips	1 tissue forceps
Small curved hemostat	Sponges
Medium size curved hemostat	
Small straight hemostat	
1 tissue forceps	
Silk	
1–5 ml syringe	
1 #25 needle (1 in.)	
3 × 3 in. sponges	

A sterile package of towels should be available for draping the arm. Sterile syringes, mineral oil and bottles for collecting blood samples are also required.

d) *Pressure-recording apparatus.*—A pressure-recording device to be connected to the catheter aids in determining the position of the catheter tip; a recording manometer (6) is preferred. If one is not available a simple saline manometer of the type generally used in venous pressure determinations suffices. It can be mounted on a stand so that the zero point on the scale is adjustable to the assumed level of the patient's right atrium.

e) *Apparatus for measuring oxygen consumption.*—The patient's oxygen consumption may be determined with a standard metabolism apparatus or by an open circuit method using Douglas bags and analyzing their contents. With the latter, a means of measuring the amount of gas in the Douglas bag (a Tissot spirometer or a gas meter) and a Haldane gas analysis apparatus are required.

f) *Apparatus for chemical analyses.*—To determine the oxygen content of the blood samples most laboratories utilize the manometric Van Slyke apparatus (9), although the Roughton-Scholander micromethod for oxygen determination has proved sufficiently accurate for cardiac output determinations (10). In England the Haldane blood-gas analyzer is used (4).

* Obtainable from U. S. Catheter and Instrument Company, Glens Falls, N. Y.

PROCEDURE

a) Preparation of patient.—The patient should be as relaxed as possible, mentally and physically, during catheterization and estimation of cardiac output. Use of the fluoroscope, necessitating operation in an x-ray room, is unfortunate, since the surroundings may make the patient apprehensive. Ideally the room should look not like a laboratory but like an attractive hospital room. The surroundings should be pleasant and quiet, especially when basal determinations are being made.

In most instances we do not use premedication. In extremely apprehensive subjects a mild sedative may be given, and in infants and small children general anesthesia may be required. We do not use an anticoagulant or an antibiotic to prevent infection. In some laboratories sulfonamide is placed in the wound or penicillin is administered systemically as a prophylactic measure.

Whether a vein of the right or of the left arm is used appears to make relatively little difference. The path of the catheter is shorter from the right side, but angulation into the superior vena cava is more acute. From the left side the catheter must pass across the upper mediastinum where occasionally, but not frequently, angulation or tortuosity of the venous channels makes passage of the catheter difficult or impossible. In either arm the median antecubital vein is the one best suited for catheterization. If the cephalic vein (near the lateral aspect of the antecubital space) is used, its entrance into the axillary vein is at approximately a right angle and passage is frequently difficult. Time and care spent in selection of the best vein is well worth while, since it may make the difference between success and failure.

b) Catheter insertion.—While the operator is scrubbing, an assistant sterilizes the skin area around the patient's elbow. After putting on rubber gloves, the operator drapes the patient's arm with sterile towels so that only a short length of arm near the proposed site of incision is exposed. It is well to place a folded towel to interrupt the patient's line of vision down the arm. The area of the incision is infiltrated with procaine and a sterile tourniquet applied above the elbow. After the anesthetic has taken effect a transverse incision about 1 cm long is made. When healed it will be less obvious if made along the transverse linear markings of the antecubital space. The vein is isolated by blunt dissection. A small hemostat is very useful in this procedure. In general, it appears that the less local trauma, the less tendency there is to venospasm. When the vein is isolated a double loop of silk is passed under it. The silk is divided and one piece pulled to the distal end of the exposed part of the vein and tied with a single knot. This prevents retrograde bleeding when the vein is opened. The other piece of silk is looped around the proximal end of the exposed vessel but is not tied. This loop serves as a means of traction on the vein.

The catheter and attachments are next prepared. In our laboratory we have connected the catheter to a 3-way stopcock, the side-arm of which

is attached by rubber tubing to a bottle of physiologic saline solution such as used for hypodermoclysis. The saline is allowed to run through the catheter to displace all air. The operator then moistens the external surface of the catheter by wiping with a wet sponge and inspects it for any blemishes or foreign matter. The vein is elevated by one of the silk ties and a small fish-mouth incision made with a bayonet type scalpel. The catheter tip is inserted in the vein and passed several centimeters up the lumen.

The fluoroscopic screen is now put in place and further passage of the catheter is carried out with the aid of x-rays. The catheter tip is passed up the arm and into the thoracic cavity. If an obstruction is met on attempting to enter the thoracic cavity, rotation of the catheter or slight movement of the arm and shoulder joint usually allows the catheter to proceed without difficulty. At no time should force be used. Once in the thoracic cavity the catheter is directed into the superior vena cava. To prevent its entrance into the neck veins, it is best to have the patient turn his head toward the side of the operator during this phase. Even so, sometimes the catheter passes into the neck veins or across the mediastinum; in this case it is withdrawn several centimeters, rotated and reinserted.

After passing down the superior vena cava the catheter enters the right atrium. It is preferable to have the catheter tip in the vicinity of the tricuspid valve for best sampling of mixed venous blood in the atrium. The appearance at this time is shown in Figure 1. If the catheter is in this position it is usually easily advanced farther into the right ventricle. When the catheter is in the tricuspid area ectopic beats may occur, and it is probably not desirable to leave the catheter in such a position. The position of the catheter when in the ventricle is shown in Figure 1. At times while in the atrium the angulated tip persistently points toward the subject's right and shows no tendency to enter the ventricle directly. In these circumstances advancement of the catheter often produces a *redundant loop, and the tip will pass into the ventricle (Fig. 1). On other* occasions the tip tends to pass into the inferior vena cava or hepatic veins; if this occurs it can be withdrawn and reinserted in the proper direction. Frequently many attempts are required before the desired location is attained.

If the catheter tip is in the right ventricle further passage into the pulmonary artery can usually be accomplished. The tip passes cephalad, then into either the right or left lung field. Care must be taken that the catheter does not proceed too far at this time if one desires to obtain samples of mixed venous blood. If the tip is too far into the finer ramifications of the pulmonary artery, arterialized blood may be withdrawn, the result of retrograde flow from pulmonary capillaries.

Samples of blood from the proximal pulmonary artery appear to be most representative of mixed venous blood. Samples from the ventricle or atrium are more variable (7). Particularly in the latter instance is the

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b) *Catheter insertion.*—While the operator is scrubbing, an assistant sterilizes the skin area around the patient's elbow. After putting on rubber gloves, the operator drapes the patient's arm with sterile towels so that only a short length of arm near the proposed site of incision is exposed. It is well to place a folded towel to interrupt the patient's line of vision down the arm. The area of the incision is infiltrated with procaine and a sterile tourniquet applied above the elbow. After the anesthetic has taken effect a transverse incision about 1 cm long is made. When healed it will be less obvious if made along the transverse linear markings of the antecubital space. The vein is isolated by blunt dissection. A small hemostat is very useful in this procedure. In general, it appears that the less local trauma, the less tendency there is to venospasm. When the vein is isolated a double loop of silk is passed under it. The silk is divided and one piece pulled to the distal end of the exposed part of the vein and tied with a single knot. This prevents retrograde bleeding when the vein is opened. The other piece of silk is looped around the proximal end of the exposed vessel but is not tied. This loop serves as a means of traction on the vein.

The catheter and attachments are next prepared. In our laboratory we have connected the catheter to a 3-way stopcock, the side-arm of which

of blood appears through the fine bore. With the needle in place, the sharp stilet is removed and replaced by a blunt solid one. If the needle is threaded up the artery a short distance it may be left in place for some time without pain or danger of clotting. Samples of blood for oxygen analysis are removed in a manner similar to that used in obtaining samples from the catheter.

d) Measurement of oxygen consumption.—This may be done by an open circuit method, collecting expired air in a Douglas bag or Tissot spirometer over a measured period. It is preferable to have an outside source for air intake, thus providing greater constancy in composition. Following a washing out period, collection of a 2 or 3 min sample appears to be adequate. The amount of air collected is measured and a small sample analyzed for oxygen and carbon dioxide content. From these data, knowledge of the composition of inspired air, temperature and barometric pressure, the amount of oxygen consumed during a unit period of time may be calculated as in metabolism studies (9).

Oxygen consumption may also be determined with an ordinary metabolism apparatus. Conventionally this means the breathing of pure oxygen. In this case the determination should be delayed until after the blood samples are collected, so that oxygen inhalation will not affect the blood levels. Use of room air in the metabolism apparatus appears possible and may avoid distortion of the blood samples.

e) Estimation of cardiac output.—This is readily obtained by the following calculations. If, for example,

$$\begin{aligned} \text{oxygen consumption} &= 250 \text{ cc/min} \\ \text{arterial } O_2 \text{ content} &= 200 \text{ cc/l} \\ \text{venous } O_2 \text{ content} &= 150 \text{ cc/l} \\ \text{cardiac output} &= \frac{250}{200 - 150} = \frac{250}{50} = 5 \text{ l/min.} \end{aligned}$$

To minimize variations due to different body sizes, it is often useful to calculate the so-called cardiac index. This is the cardiac output in l/min/sq m body surface area.

Whenever it is felt that the cardiac output may be rapidly changing, sampling of blood and of expired air should be done simultaneously. We believe that when dealing with relatively stable states, however, sampling in rapid succession has certain advantages. The venous sample is obtained first because it disturbs the patient least. This is followed rapidly by the arterial sample and finally by the oxygen consumption measurement. The last is most likely to disturb the patient, and if this does occur only one value rather than two or three will be distorted.

f) Catheter removal.—On completion of the procedure and removal of the catheter the two silk ties around the vein are also taken out. The skin is closed with two fine silk sutures, and any bleeding controlled by a pressure bandage. The sutures are removed several days later, and in most instances the vein will be recanalized in a few weeks.

Critique.—The direct Fick principle applies in nearly all situations. It

operator liable to error because of inadequate mixing of the venous blood (13). An additional source of error is entrance of the catheter into the coronary sinus. Blood from this vessel has an extremely low oxygen content and is, of course, not representative of mixed venous blood (1).

c) Blood samples.—With the catheter in place, sampling of the blood can be carried out. The slow drip of saline is turned off and several milliliters of blood withdrawn and discarded to flush out the saline solu-

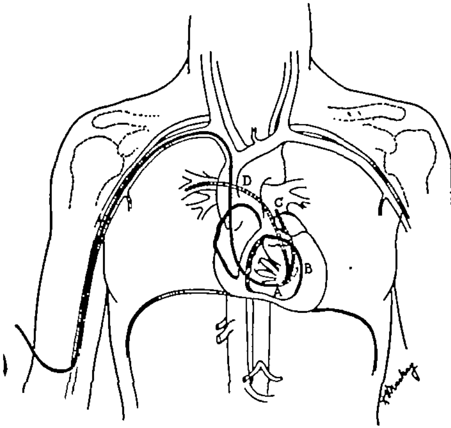

FIG. 1.—Position of catheter in: *A*, region of tricuspid valve; *B*, right ventricle; *C*, proximal pulmonary artery; *D*, distal pulmonary artery.

tion in the catheter system. Pure blood samples are then withdrawn into a syringe under oil or over mercury and transferred to suitable containers under oil or over mercury.

Arterial blood is obtained by direct puncture. We usually use the femoral artery just distal to the inguinal ligament. The skin area and subcutaneous tissue around the site of puncture are anesthetized with procaine. A special needle† has proved most satisfactory. Continuation of the needle tubing through the hub prevents contamination of samples by clotted blood contained in the hub. The needle is inserted with its sharp open-bore stilet in place. When the artery is entered a small drop

† Cournand needle, made by Becton-Dickinson & Co., Rutherford, N. J.

of blood appears through the fine bore. With the needle in place, the sharp stilet is removed and replaced by a blunt solid one. If the needle is threaded up the artery a short distance it may be left in place for some time without pain or danger of clotting. Samples of blood for oxygen analysis are removed in a manner similar to that used in obtaining samples from the catheter.

d) Measurement of oxygen consumption.—This may be done by an open circuit method, collecting expired air in a Douglas bag or Tissot spirometer over a measured period. It is preferable to have an outside source for air intake, thus providing greater constancy in composition. Following a washing out period, collection of a 2 or 3 min sample appears to be adequate. The amount of air collected is measured and a small sample analyzed for oxygen and carbon dioxide content. From these data, knowledge of the composition of inspired air, temperature and barometric pressure, the amount of oxygen consumed during a unit period of time may be calculated as in metabolism studies (9).

Oxygen consumption may also be determined with an ordinary metabolism apparatus. Conventionally this means the breathing of pure oxygen. In this case the determination should be delayed until after the blood samples are collected, so that oxygen inhalation will not affect the blood levels. Use of room air in the metabolism apparatus appears possible and may avoid distortion of the blood samples.

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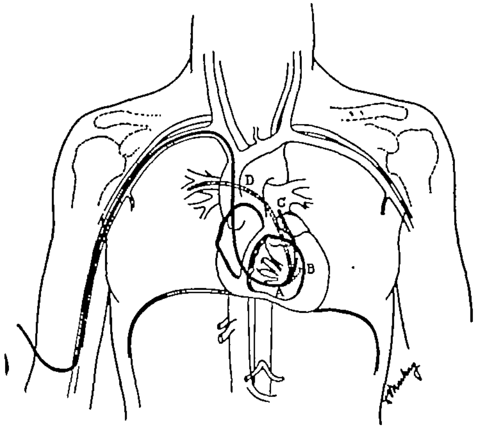

FIG. 1.—Position of catheter in: *A*, region of tricuspid valve; *B*, right ventricle; *C*, proximal pulmonary artery; *D*, distal pulmonary artery.

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assure greater safety to the catheterization technique; (2) to discuss errors inherent in determination of cardiac output by the direct Fick procedure.

To assure safety it is essential that (a) all displacements of the catheter tip in the right heart be performed under direct fluoroscopic control; buckles of the catheter as it progresses in the venous system and loops should be avoided. (b) Displacement of the catheter tip from the right auricle to the right ventricle and between the pulmonary artery and the outflow track of the right ventricle should always be done under continuous electrocardiographic control. Premature contractions are often observed as the catheter tip moves through the tricuspid ring or in the outflow track of the right ventricle. If premature contractions persist, the catheter should be displaced without delay and withdrawn eventually to the right auricle. It is often observed that, during sampling of blood from the right ventricle, ectopic beats occur if blood does not flow freely under slight negative pressure in the syringe. Therefore the catheter tip should always be *located in a position that allows free and easy sampling.* Ectopic beats are more apt to occur in cardiac patients, and in a few instances very short runs of ventricular tachycardia have been observed. Therefore in these patients it is better to play safe by withdrawing samples from the right auricle if the catheter in the right ventricle causes ectopic beats. It is recommended, in addition, that the pulse rate be checked frequently and the catheter be withdrawn if it increases significantly. In summary, as long as the catheter is in the auricle, disturbances of rhythm are most unusual. With the development of right ventricular or pulmonary artery catheterization, potential dangers have certainly increased, therefore the necessity for greater precautions.

As to accuracy of the method, there is no doubt that sampling from the pulmonary artery and outflow track of the right ventricle in subjects without cardiac shunts gives greater assurance that the sample is representative of completely mixed venous blood. However, right auricular blood samples will check within 0.2 vol per cent with the pulmonary artery sample in 75 per cent of cases. With regard to constancy of results, it has been our experience that, provided proper care is taken in the handling of the patient, large variations in blood flow over a long period may be minimized in the resting state. Summarizing recent work in this laboratory, Lars Werko (The influence of positive pressure breathing on circulation in man, Acta med. Scandinav., supp., 1947) stated that in repeated determinations of cardiac output in 20 subjects, with an interval of at least one hour between determinations, the cardiac output figures checked within 5 per cent in eight cases and within 15 per cent in 28. The total error of the Fick method seldom exceeds 15 per cent—a figure obtained by summation of the technical errors in blood-gas analysis and in determination of oxygen consumption and of less tangible factors.

Finally, the details of the procedure are explained to the patients, and in adhering to this policy good co-operation is usually secured and no cause for anxiety created.

Comment by Harold D. Green

For data on cardiac output in the dog obtained with the catheterization technique, see Shore, R.; Holt, J. P., and Knoefel, P. K.: Determination of cardiac output in the dog by the Fick procedure, Am. J. Physiol. 143: 709–714, 1945.

is not valid in the presence of certain congenital heart lesions such as atrial septal defect, in which mixing of right and left heart blood occurs. Aside from those rare anomalies the principle appears to be valid, but there are several sources of error. The adequacy of the mixing of venous blood in the atrium has already been discussed. Blood from the ventricle and particularly the proximal pulmonary artery appears to be more representative of mixed venous blood. All oxygen measurements, both blood and gas analyses, are subject to some error. The net result of these small errors and the error in mixing may create a quite large final error in the cardiac output determination. This problem has been discussed in greater detail elsewhere (3, 13).

Comment by André F. Cournand

The technique used in the Cardio-Pulmonary Laboratory of the Chest Service at Bellevue Hospital corresponds in general to that described in the preceding pages. Minor differences in details are listed here:

1. Since better records of blood pressure are obtained with no. 6 and 7 catheters than with no. 8 and 9 sizes, the latter sizes have been discarded. No difference in ease of sampling has been noted.
2. In choosing a vein for introduction of the catheter in the venous system, it is important to maintain continuous venous flow around the catheter once it is introduced in the brachial venous system. Therefore the ideal site of introduction is below the confluence of the median basilic vein with the other branches running medially to it in the forearm.
3. For exposure of the vein a very small incision is made in the line of the vein, not transverse to its diameter. As a result no suture is required when the procedure is over.
4. The two silk ties passed around the exposed vein are always removed after the catheter has been withdrawn.
5. After removal of the catheter, care should be taken to avoid stasis in the brachial vein which may follow tight bandaging. The patient is urged to move his arm to restore adequate venous flow. This is especially important in patients with cardiac decompensation and polycythemia.
6. The arterial samples are drawn from an indwelling needle placed in the brachial rather than the femoral artery.
7. The syringes used for blood sampling are prepared in the following manner. The plungers are greased lightly; several drops of a heparin solution (400 mg of NaF added to 3 ml of Liquaemin [Roche-Organon] and 3 ml of distilled water) and a small drop of mercury are introduced in the syringe, and with the syringe in the upright position the plunger is advanced completely. In this way all air is displaced from the syringe and the solution remaining in the tip serves as an anticoagulant. Mixing is facilitated by the droplet of mercury.
8. After blood samples are drawn, the syringes are plugged with round toothpicks and rotated slowly to prevent settling of cells. Blood is transferred directly under pressure from the syringes to 1 ml Ostwald-Van Slyke pipets by means of a 20 gauge needle inserted in the tip of the pipet. A small piece of rubber, pierced by the needle, makes an air-tight seal during the transfer.

The following remarks are intended (1) to emphasize essential precautions to

Theoretically such a device has long been a possibility, based on a combination of a d-c type electronic amplifier with either an ionization chamber or a photoelectric cell and fluorescent screen. But both of these sources necessitate extremely high-gain d-c amplifiers that are notoriously unstable. Early attempts demonstrated the feasibility of roentgen electrokymography, but until recently a practical instrument was not forthcoming.

First practical application came with reports of successful use of the multiplier phototube (R.C.A. no. 931-A and similar types) (10) in measurement of x-ray intensity in the efferent roentgenographic beam (exposure meter (7)) and in the automatic termination of the roentgenographic exposure (phototimer (8, 9)). In 1944 Henny, Boone and Chamberlain, following a direct suggestion by R. H. Morgan, produced a

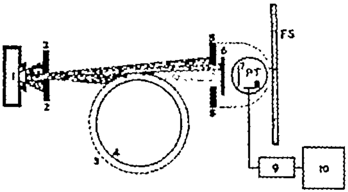

FIG. 1.—Electrokymograph applied to heart shadow border. *1*, x-ray tube of conventional fluoroscope; *2*, shutter blades of fluoroscope; *3*, cross-section of heart in diastole; *4*, cross-section of heart in systole; *5*, aperture, metallic lead; *6*, small piece of fluorescent screen; *PT*, 931-A (or 1 P 21) multiplier phototube; 7, photosensitive cathode of phototube; *8*, collector electrode (anode) of phototube; *9*, "filter" (see text); *10*, electrocardiograph, used as recording galvanometer; *FS*, fluoroscope screen, used for centering pick-up (*5*, *6*, *PT*) over selected part of heart shadow.

practical device based on a combination of the 931-A phototube, a bit of type B Patterson fluoroscopic screen and a recording galvanometer (2, 5, 6).

APPARATUS

The apparatus consists essentially of the clinical fluoroscope, multiplier phototube with its power supply, a bit of fluorescent screen closely applied over the window of the phototube, a filter circuit for attenuation of the alternating current ripple in the fluoroscopic x-ray beam, the recording galvanometer (any satisfactory electrocardiograph) and means for recording simultaneously, as a time-reference curve, an arterial pulse tracing, a heart sound oscillogram or an electrocardiogram (Figs. 1 and 2).

Any clinical *fluoroscope* may be used, but one with a full-wave, 4-valve tube-rectified power supply and cable-connected shockproof x-ray tube

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VI. Roentgen Electrokymograph*

M. J. OPPENHEIMER *and* W. EDWARD CHAMBERLAIN, *Temple University*

The chief use for the roentgen electrokymograph is in production of graphic records of shadow border motion and density changes in a fluoroscopic image. As the heart and great vessels go through their systolic-diastolic cycle, two types of associated change take place in the fluoroscopic shadow image: (*a*) motion of shadow borders (e.g., left border of ventricular shadow moves dextrad during systole, sinistrad during diastole); (*b*) intensity change, owing to the fact that x-ray transmission through the pulsating organ is decreased while the part is full, increased while it is contracted (dorsoventral thickness decreased). With the electrokymograph these changes in the fluoroscopic shadow image are converted into variations in an electric current and recorded by any suitable recording galvanometer.

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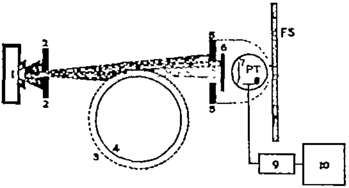
* Development of the roentgen electrokymograph and its application to studies of normal and morbid physiology has been a co-operative enterprise of the Heart Disease Control Section, U.S. Public Health Service and the Departments of Physiology (Research Grant 194, N.I.H.), Radiology and Medical Physics.

Theoretically such a device has long been a possibility, based on a combination of a d-c type electronic amplifier with either an ionization chamber or a photoelectric cell and fluorescent screen. But both of these sources necessitate extremely high-gain d-c amplifiers that are notoriously unstable. Early attempts demonstrated the feasibility of roentgen electrokymography, but until recently a practical instrument was not forthcoming.

First practical application came with reports of successful use of the multiplier phototube (R.C.A. no. 931–A and similar types) (10) in measurement of x-ray intensity in the efferent roentgenographic beam (exposure meter (7)) and in the automatic termination of the roentgenographic exposure (phototimer (8, 9)). In 1944 Henny, Boone and Chamberlain, following a direct suggestion by R. H. Morgan, produced a



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practical device based on a combination of the 931–A phototube, a bit of type B Patterson fluoroscopic screen and a *recording galvanometer* (2, 5, 6).

APPARATUS

The apparatus consists essentially of the clinical fluoroscope, multiplier phototube with its power supply, a bit of fluorescent screen closely applied over the window of the phototube, a filter circuit for attenuation of the alternating current ripple in the fluoroscopic x-ray beam, the recording galvanometer (any satisfactory electrocardiograph) and means for recording simultaneously, as a time-reference curve, an arterial pulse tracing, a heart sound oscillogram or an electrocardiogram (Figs. 1 and 2).

Any clinical *fluoroscope* may be used, but one with a full-wave, 4-valve tube-rectified power supply and cable-connected shockproof x-ray tube

has distinct advantages (see later discussion of design of filter). Current and voltage settings ordinarily used for clinical fluoroscopy, e.g., 3 ma at 85 kv peak, are entirely adequate.

The R.C.A. no. 931-A multiplier *phototube* (Figs. 1–3) consists of a photoelectric surface and nine stages of electron multiplication. Electrons emitted by the cathode (photoelectric surface) as a result of bombard-

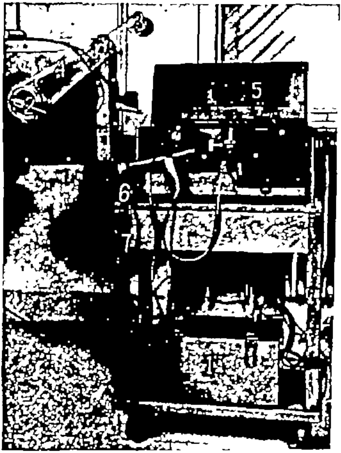

FIG. 2.—Electrokymograph with pick-up mounted at center of fluoroscopic screen. *1*, carrying case containing power pack, filter, etc.; *2*, pick-up housing which encloses multiplier phototube, metallic lead diaphragm with 5 × 20 mm aperture (visible in photograph), small piece of fluorescent screen and resistance divider network (*R1–R10* in Fig. 3); *3*, pulley for belt drive which permits operator to rotate pick-up without approaching x-ray beam; *4*, arm, of plywood or plastic; *5*, electrocardiograph (recording galvanometer); *6*, cup of carotid pulse neckpiece; *7*, rubber tubing for conducting pulse wave to recorder.

ment by photons are accelerated toward the first dynode by a potential difference of approximately 100 v. For each electron which thus bombards the first dynode (energy, 100 electron volts), several electrons are emitted and accelerated toward the second dynode where the process of electron multiplication is duplicated. There are nine of these dynodes, and the current finally registered at the collector electrode is the original photoelectric emission multiplied by the ninth power of the electron

multiplication factor. The power supply may be any source of constant potential of approximately 1000 v. A satisfactory circuit is shown in Figure 8. The resistance network voltage-divider (*R1–R10*) assigns the correct potential difference to consecutive dynodes; it is small and easily accommodated in the phototube housing.

The bit of *fluorescent screen* closely applied over the window of the phototube must be practically free from lag, for obvious reasons. Green-fluorescing Patterson type B screen is eminently satisfactory. We have also used an experimental Patterson screen, no. Q 20–774 C, which emits light of a color particularly suited to the spectral response of the 931–A phototube.

X-ray sources of clinical fluoroscopy are customarily of two types: (1) half-wave, usually self-rectified, meaning that the high voltage transformer output is connected directly to the terminals of the x-ray tube, which acts as its own rectifier since it will pass current in only one direction; (2) full-wave, meaning that the high voltage transformer output passes through a 4-valve rectifier on its way to the x-ray tube. Commercially available alternating current is usually of 60 c frequency. Thus the fluoroscopic x-ray beam is composed of pulses, 60/sec for the half-wave circuit, 120/sec for full-wave rectification. Because of this ripple in output of the x-ray tube, a highly selective "filter" must be interposed between phototube and galvanometer. Without such a filter the tracings would be blurred, since the galvanometer would follow the rapid fluctuations in x-ray output.

The *filter* must be sufficiently selective to remove undesirable fluctuations without destroying the fidelity of the electrokymographic record. This is easier to accomplish in the full-wave rectified, cable-connected, shockproof fluoroscope (120 pulses/sec) than in the self-rectified type (60 pulses/sec) for two reasons. (*a*) At customary current settings, of the order of 3 ma, capacitance of the insulated and shielded cables that conduct the high voltage current from transformer to x-ray tube minimizes undesirable fluctuations in x-ray output by suppressing a high percentage of voltage fluctuation at the tube terminals. (*b*) The cardiodynamic phenomena being recorded exhibit frequencies of the order of 1–25 c/sec. To record these phenomena with complete fidelity, the filter must give zero transmission at the unwanted frequency of the ripple in the x-ray beam and no selective absorption at the frequencies of the physiologic processes. It is easier to achieve this for a 120 pulse ripple than for a 60 pulse ripple since the latter is much closer to the maximal frequencies that may be encountered in these physical processes.

Any satisfactory electrocardiograph may be used as the recording galvanometer. We have had much satisfaction from the type that utilizes an Einthoven string galvanometer (Simplitrol, Cambridge Instrument Company), but the type which uses a mirror and light beam galvanometer of the d'Arsonval type, fed from a power amplifier (e.g., the Sanborn Cardiette), is equally adaptable. To some extent the types are inter-

changeable, since it is simple to substitute one for the other in any of the circuits we have worked with. One major difference should be noted. With the Cambridge device, sphygmograms are obtained with a tambour and pointer. With the Sanborn, a high sensitivity galvanometer channel

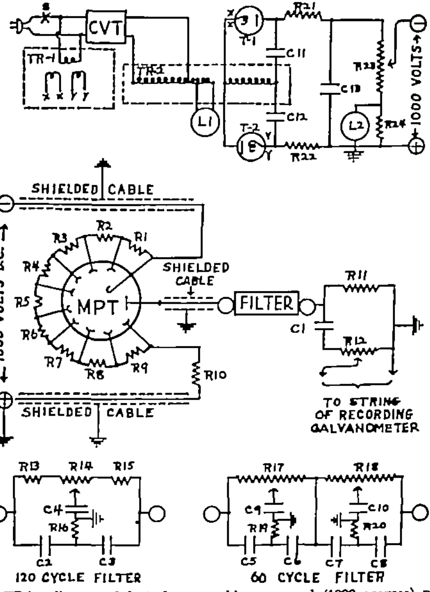

FIG. 3.—Wiring diagram of electrokymographic power pack (1000 v source), pick-up and two types of filter. (Specifications given in text.)

(in addition to the one used for the electrokymogram) is required for the sphygmogram.

The circuit for electrokymography when the recording galvanometer is a Cambridge Simplitrol is shown in Figure 3. The specifications are as follows for operation from an a-c source of 110–120 v: *s* = power control switch. *CVT* = Sola constant voltage (stabilizer) transformer, 301,003, 15 w. *TR-1* = SNC (SNC Manufacturing Company, Glenview, Ill.)

4 P 222 dual filament, 2.5 v transformer. *TR-2* = SNC 8 P 186 power transformer, 325–0–325 v. *L1* = 6 v pilot lamp (low candle power). *L2* = GE $^3/_4$ w, 125 v neon lamp. *T-1* and *T-2* = 2 X 2 (879) rectifier tubes. *R21* = 0.2 megohm, 2 w resistor. *R22* = 0.1 megohm, 2 w resistor. *R23* = 2 w potentiometer of approximately 1.7 megohm, adjusted to give output of 1000 v to phototube network. (*R23* should not be of the knob and dial type, which invites frequent readjustment; the type adjusted by moving a contact band to a fixed position is preferred.) *R24* = 150,000 ohms, 2 w. *C11* and *C12* = 1 microfarad, 1000 v, and *C13* = 2 microfarad, 1500 v condensers. *MPT* = no. 931–A (or 1 P 21) multiplier phototube. *R1–R9* = 0.1 megohm, $^1/_2$ w resistors. *R10* = 50,000 ohms, $^1/_2$ w. *R11* = 1 megohm, $^1/_2$ w. *R12* = 10,000 ohm potentiometer (carbon), linear taper. *C1* = 2 microfarad paper condenser.

The filters are the "parallel-T" type. Formulae for zero transmission at stipulated frequencies are given in *Radio Engineers' Handbook*.† *R13* and *R15* = 10,000 ohms, $^1/_2$ w. *R14* = 20,000 ohm potentiometer (carbon), linear taper. *C2* and *C3* = 0.05 microfarad paper condensers. *C4* = 0.18 microfarad paper. *R16* = 17,500 ohms, $^1/_4$ w. *R17* and *R18* = 100,000 ohm potentiometers (carbon), linear taper. *R19* and *R20* = 7000 ohms, $^1/_4$ w. *C5*, *C6*, *C7* and *C8* = 0.1 microfarad, 400 v paper condensers. *C9* and *C10* = 0.06 microfarad, 400 v paper condensers.

Various modifications of the circuit and specifications will occur to anyone familiar with electronic amplifiers and phototubes. For example, in some applications the interposition of one stage of vacuum tube amplification immediately before the string galvanometer might prove advantageous. B. R. Boone is investigating variations in the arrangement for coupling the phototube output with the galvanometer (or amplifier).

The blocking condenser (*C1*) is essential. Without it the galvanometer would record the d-c component of the signal instead of only the significant fluctuations. But the resistances, *R11* and *R12*, must have correct values for the required time constant. If this time constant is too long, valuable time will be lost at the beginning of each record because suppression of the d-c component of the signal will take place too slowly; if it is too short, fidelity will be lost (e.g., horizontal segments in the electrokymogram will be reproduced as slopes toward the base line). The values given for *C1* (2 microfarads), *R11* (1 megohm) and *R12* (10,000 ohm potentiometer) have proved satisfactory in many applications. However, Boone has found apparent advantages in quite different values. *C1* may be given a value of 10 microfarads, in which case a paper condenser becomes inconveniently bulky and an electrolytic condenser may be substituted; one of 10 microfarad capacitance may be used. It should be connected with its negative pole toward *R11*, positive toward *R12*. The value of *R11* must now be changed, and a variable element is sug-

† *Radio Engineers' Handbook* (New York: McGraw-Hill Book Company, Inc., 1943), pp. 905 and 918.

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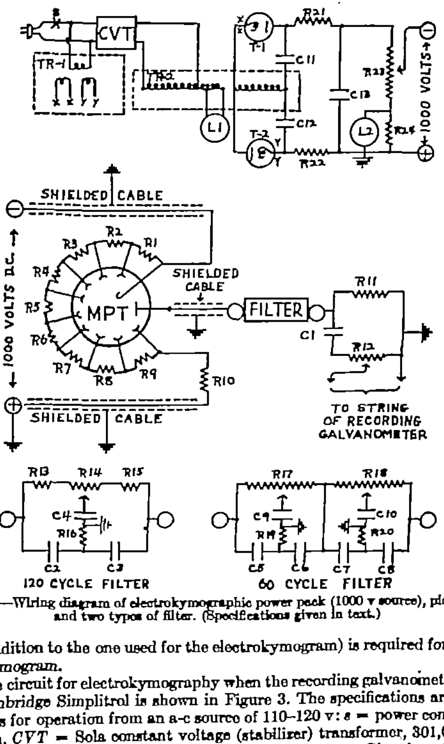

FIG. 3.—Wiring diagram of electrokymographic power pack (1000 v source), pick-up and two types of filter. (Specifications given in text.)

(in addition to the one used for the electrokymogram) is required for the sphygmogram.

The circuit for electrokymography when the recording galvanometer is a Cambridge Simplitrol is shown in Figure 3. The specifications are as follows for operation from an a-c source of 110–120 v: *s* = power control switch. *CVT* = Sola constant voltage (stabilizer) transformer, 301,003, 15 w. *TR-1* = SNC (SNC Manufacturing Company, Glenview, Ill.)

aorta, pulmonary artery, vena cava, in various angles of projection. Records of thickness change are readily obtainable by alining the aperture of the pick-up with an appropriate shadow area, instead of with the shadow border (Fig. 5). Applications in the field of cardiovascular physiology are obvious. The method also is applicable in the clinical field. It has been used to study asynchronism of ejection of the ventricles in normal subjects and patients with bundle-branch block (1, 3, 4). It will

FIG. 5.—Fluoroscopic screen image showing three of many possible positions for electrokymographic aperture. Two are placed for shadow border motion (aortic knob and left ventricle). The other is placed to record thickness changes, but is too high for a good tracing of left ventricle; in position shown it would record the composite of the thickness changes of left ventricle, outflow tract of right ventricle and left auricle.

probably prove of value in study of myocardial infarction. Its place in the study of other types of cardiovascular abnormality is still uncertain.

NOTE.—This section was reviewed by Russell H. Morgan.

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gested, to permit operation at various values between 50,000 and 100,000 ohms.

For identification of the various phases of heart action we used simultaneously recorded heart sounds, electrocardiograms and carotid pulse tracings. Figure 4 shows all three, with simultaneous electrokymograms at two points on the left border of the left ventricle. For such polybeam records we use double-width (12 cm) paper in a special camera, with three Cambridge Simplitrol string galvanometers, a Cambridge stethograph for recording heart sounds and our own model of carotid-pulse recorder, mounted in the lens tube of one of the Simplitrol galvanometer units.

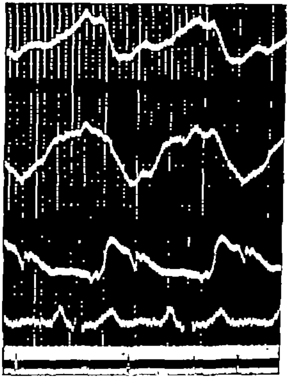

FIG. 4.—Electrokymograms of two different portions of left ventricular shadow border, with simultaneously recorded carotid pulse, electrocardiogram and heart sound oscillogram.

Such polybeam records are highly informative, although valid and useful results are obtainable with more conventional equipment. When one is limited to a single galvanometer track plus the carotid pulse record (with or without a stethogram), the electrokymographic pick-up (Fig. 2, *B*) is moved about until tracings of all desired heart chambers and great vessels have been obtained. Time relationships of these several electrokymograms to each other are then established indirectly, by reference to the simultaneously recorded sphygmograms.

Critique.—Excellent and informative electrokymograms may be obtained from shadow border motion of the several cardiac chambers,

ANALYSIS OF CARDIOVASCULAR ACTIVITY

HAROLD D. GREEN

I. Vasomotor Tone

ESTIMATES OF CHANGE of vasomotor tone are of prime importance for investigative studies since they are the basis for the detection of reflexly induced alterations in vasomotor nerve activity, appearance of circulating vasodepressor or vasodilator substances, effectiveness of tissue extracts and the analysis of the action of various synthetically produced substances. These may conceivably alter the tone of the arteries, arterioles, capillaries, venules and veins.

In a system in which flow is occurring the drop in pressure per unit length of vessel is a measure of resistance to flow in this vessel. In the smallest caliber arteries in which a cannula made of capillary glass tubing could be inserted, i.e., the distributing arteries in the mesentery as they begin to encircle the gut, we found mean pressure to be not more than a few mm Hg less than that in the aorta (11). In view of this, most of the resistance and therefore most of the changes in caliber of the vessels which can affect peripheral resistance must be in vessels with internal diameters less than 1 mm. On the other hand, intracapillary pressure appears to be of the order of 15–30 mm Hg (50, 66). For this reason measurements of change of blood flow with respect to the supplying arterial head of pressure or the arteriovenous difference of pressure probably estimate principally the activity of the vessels of diameters smaller than 1 mm which are proximal to the capillaries, i.e., peripheral vasomotor tone.

Although changes in capillary and venous tone may have some influence on the flow through the arterioles by raising or lowering intracapillary pressure, they are probably more important in influencing peripheral storage of blood and therefore the quantity available for distention of the central venous reservoir. Changes in flow are also due to variations in extravascular pressure (27, 33, 35, 37, 74) and to alterations in blood viscosity. Few quantitative data are available regarding the influence of the latter in physiologic systems (19, 26, 54, 73).

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particular disturbance of homeostasis leads simultaneously to decreased cardiac output and increased peripheral resistance, the change in peripheral vasomotor tone may be completely masked. An example was seen in studying the vascular responses to stimulation of the motor cortex. Under a barbiturate anesthetic no change in mean arterial occurred, although marked increase in limb volume due to peripheral vasodilatation was recorded. Under ether anesthesia a rise in arterial pressure occurred, which suggested vasoconstriction, although again an increase in limb volume, suggestive of peripheral vasodilatation, was observed in the hindextremity (29, 40).

More suitable is simultaneous measurement of arterial pressure and cardiac output. Under these conditions a change in cardiac output unaccompanied by an alteration in arterial pressure or, vice versa, an alteration in arterial pressure without change in cardiac output is readily interpretable as indicating a modification of total peripheral resistance caused, if viscosity is unchanged, by an alteration in vascular tone. However, not infrequently both output and resistance change and interpretation then becomes difficult. Under these conditions it is commonly assumed, if cardiac output and arterial pressure vary proportionately and viscosity has not changed, that no alteration in vasomotor tone occurred. However, no experimental data are available to indicate what alteration in total peripheral blood flow (cardiac output) would be expected to be associated with a given change in level of arterial pressure in the absence of change of vasomotor tone. Lacking such information it is probably better to indicate that peripheral resistance, defined as the ratio of arterial pressure to blood flow (see footnote *) has changed. Interpretation regarding change of vasomotor tone may be deferred until information on the anticipated alteration in peripheral resistance accompanying the recorded shift in mean arterial pressure in the absence of change in vasomotor tone becomes available.

Measurement of blood flow in isolated regions while they are perfused from the aorta has been used to interpret reflex and humoral responses to disturbance of homeostasis. Measurement of flow while the region is perfused from the animal's own circulation allows interpretation of both humoral and nervous mechanisms but is complicated, as already indicated, by difficulties in interpretation if both blood flow and level of the arterial pressure change. The significance of this has been indicated in several reports. For instance, in skin and muscle peripheral resistance in-

* Total peripheral resistance $= \frac{MAP}{CO} = TPR$; $\frac{1}{TPR} = \frac{F_1}{MAP} + \frac{F_2}{MAP} + \frac{F_3}{MAP}$, etc.; $\frac{1}{TPR} = \frac{1}{PR_1} + \frac{1}{PR_2} + \frac{1}{PR_3}$, etc., where MAP is mean arterial pressure; CO, cardiac output; F_1, F_2, etc., flow in the various vascular beds, and PR_1, PR_2, etc., peripheral resistance in the various vascular beds. Satisfactory units for resistance are $PRU_m = \frac{\text{mm Hg}}{\text{ml/min}}$ and $PRU_s = \frac{\text{mm Hg}}{\text{ml/sec}}$ and $PRU_{m} = \frac{\text{mm Hg}}{\text{ml/min/100g}}$. Nothing is to be gained by converting to so-called absolute units, i.e., $\frac{\text{dynes}}{\text{ml/min}}$ (33).

II. Total Vasomotor Tone

By producing maximal relaxation of the vessels *controlling flow*, total vasomotor tone may be estimated. In the case of skeletal and cardiac muscle this may be accomplished (*a*) by inducing maximal metabolic activity (18, 28, 31), (*b*) by a period of ischemia (18, 28, 31), (*c*) by intra-arterial injection of sodium cyanide (31) or (*d*) by reduction of blood O_2 content (18, 28, 31). These techniques are of little use in the case of skin and may lead to decreased blood flow in the kidneys (60–62). Flow in skin (2, 21), muscle (2, 43) and kidney (43) is increased by local warming and by eating, particularly of high protein foods (56), and reduced by local cooling (55).

III. Neurogenic Vasomotor Tone

The methods most commonly used to interrupt the train of vasomotor nerve impulses and thus to determine from the resulting change in blood flow the tonic activity of the vasomotor nerves are: (*a*) peripheral nerve block by cooling, nerve section or injection of a suitable anesthetic (2, 32, 33, 57, 59); (*b*) higher block at the sympathetic ganglia or spinal cord, usually by injection of procaine (2, 58); (*c*) body warming or general anesthesia to induce maximal vasodilatation in the skin (2, 4, 6, 21, 32, 59); (*d*) intravenous injection of pyrogenic substances to produce maximal vasodilatation in skin and apparently also in kidney (25), and (*e*) intravenous or intramuscular injection of tetraethylammonium chloride (etamon) to produce block of all autonomic ganglia (7, 12a, 15, 41, 49, 52) or one of the sympatholytic agents, 2-benzyl-4,5-imidazoline HCl (priscol) or N,N-dibenzyl-β-chloroethylamine (dibenamine) (1, 30, 36, 52, 68, 71, 70). In our hands etamon appears to be less effective than body warming in producing cutaneous vasodilatation. In fact cooling (presumably owing to cutaneous vasoconstriction) has occasionally followed an injection of etamon (30). Cooling, epinephrine and smoking have been used to induce vasoconstriction (67, 78).

IV. Reflex and Humoral Responses to Disturbances of Homeostasis

Registration of arterial pressure, either mean or systolic and diastolic, is most commonly used. Unfortunately, in such conditions alterations in arterial pressure result from changes in cardiac output and total peripheral resistance;* and the latter may be due to alterations of blood viscosity and extravascular pressure as well as to vasomotor tone. If the

* See footnote, page 243.

fer to use the unanesthetized animal (24) (see p. 252); others prefer anesthetized or even pithed animals (20, 39, 64, 65, 69). In our experience these methods have two distinct disadvantages. Frequently the given preparation, say a vasodilator drug, causes peripheral vasodilatation and simultaneous increase in cardiac output. As a result the change in mean arterial pressure is minimized and may actually be completely masked. Furthermore, the magnitude of the response alters frequently throughout an experiment, presumably owing to variations in depth of the anesthesia and to alterations in the intrinsic vasomotor tone accompanying spontaneous fluctuations in cardiac output and mean arterial pressure. Also, the quantity of the substance needed is often considerable. The method has been most commonly used in bioassay of the humoral substances associated with renal hypertension, and for this purpose nephrectomized animals have often been employed (10, 39, 65, 72). The apparent success in this condition may be due to a minimal effect of the hypertensive substances on cardiac output (51). Depressor materials which we have been studying have not had this fortuitous property (34).

To obviate the foregoing disadvantages numerous perfusion techniques have been employed. Among these are perfusion of the ear (p. 123), perfusion of the hindlegs of the frog (p. 129), microscopic observation of capillaries in the mesentery (p. 131) (47) and ear (p. 139), perfusion of the isolated beating heart (3, 14) and registration of the contraction of isolated rings of the blood vessels and perfusion of isolated extremities (9, 23).

To avoid the complications associated with use of blood, saline solutions have been employed, particularly in the isolated heart and in the frog preparation. It is our impression that, although these may be suitable for study of vasoconstrictor agents, solutions of low oxygen content probably lead to maximal vasodilatation and render the preparations less suitable for study of vasodilator substances. For the latter we believe blood perfusion is essential and prefer to use a preparation (see Fig. 3, p. 122) in which the animal serves as the perfusion system and the tissue, if possible, retains its normal innervation so as to possess a normal degree of vasomotor tone. Demonstration of adequate vasodilator response in any preparation used should accompany a negative response to an unknown substance before the latter should be considered to be lacking in vasodilator power. In addition it should be remembered that pyrogens produced by even a few hours' bacterial growth cause marked vasodilator responses in most vascular beds. During study of vasodilator substances scrupulous care must be taken to avoid bacterial growth during their preparation (23).

The use of certain chemical agents may help unmask the activity of some preparations. For instance, in our experience, etamon masked the response to a vasodilator agent which we were studying but accentuated

creases with decline in arterial pressure under conditions in which neurogenic and humorally controlled vasomotor tones are believed to remain constant (33, 54). In the kidney the reverse is thought to be true by some (13, 22, 63) but doubted by others (5). These observations emphasize the necessity for knowledge of the anticipated change in peripheral resistance which might be due to extraneous factors before concluding that a given change in resistance is due to an alteration in vasomotor tone induced reflexly or by humoral mechanisms.

Perfusion of a vascular bed at a constant head of pressure (see p. 119) obviates the difficulty and is suitable, therefore, for study of neurogenic vasomotor responses but is, of course, less suitable for study of humoral mechanisms. Interpretation under these conditions is complicated in the presence of concomitant changes in level of the arterial pressure in the animal caused by variable exchange of blood between cognate and collateral vascular beds unless suitable steps are taken to isolate adequately the cognate bed (see p. 218).

In most regions mean flow registration is satisfactory for interpretation of vasomotor activity. Variations in vasomotor activity in the cardiac vascular bed cannot be determined accurately from mean flow data because the variable effects of duration of systole and of extravascular compression during systole and the first half of diastole may alter mean flow in the absence of change of vasomotor activity and minimize or reverse a change due to vasomotor activity. The only safe measure is registration of the instantaneous inflow at the moment of onset of isometric contraction (28, 31). This can be accomplished only with instantaneous flow meters (see pp. 101 and 108).

Direct observation of the blood vessels provides interesting data regarding those portions of the vascular bed which participate in the observed responses (see p. 131). The method suffers from two disadvantages: (a) it is possible to study only relatively thin structures, which may not be characteristic of the body as a whole, and (b) rarely has microscopic observation been combined with simultaneous observation of blood flow or of arterial pressure. Such a combination would considerably improve the significance of the observations and resulting conclusions.

V. Bioassay of Tissue Extracts, Drugs and Synthetic Substances with Vascular Activity

The degree of alteration of mean arterial pressure is usually chosen as a measure of the vascular effect of a given substance. The activity of the unknown is compared with that of a standard quantity of a known substance, or various concentrations of the unknown are injected until a response equivalent to that of the known is obtained. Some authors pre-

fer to use the unanesthetized animal (24) (see p. 252); others prefer anesthetized or even pithed animals (20, 39, 64, 65, 69). In our experience these methods have two distinct disadvantages. Frequently the given preparation, say a vasodilator drug, causes peripheral vasodilatation and simultaneous increase in cardiac output. As a result the change in mean arterial pressure is minimized and may actually be completely masked. Furthermore, the magnitude of the response alters frequently throughout an experiment, presumably owing to variations in depth of the anesthesia and to alterations in the intrinsic vasomotor tone accompanying spontaneous fluctuations in cardiac output and mean arterial pressure. Also, the quantity of the substance needed is often considerable. The method has been most commonly used in bioassay of the humoral substances associated with renal hypertension, and for this purpose nephrectomized animals have often been employed (10, 39, 65, 72). The apparent success in this condition may be due to a minimal effect of the hypertensive substances on cardiac output (51). Depressor materials which we have been studying have not had this fortuitous property (34).

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the mean arterial pressure response to epinephrine, whereas priscol abolished the response to epinephrine, i.e., the vasopressor response, but accentuated the depressor response to our vasodilator substance. In other preparations removal of a pressor substance by boiling or by dialysis unmasked the presence of a depressor or vasodilator material (48, 1, 7, 15, 16, 30, 36, 41, 49, 52, 68, 71, 79).

VI. Expression of Unitage of Chemical Substances

The amount of renin which causes a rise in mean arterial pressure of 30 mm Hg within 3 min on injection into an unanesthetized dog weighing between 10 and 25 kg has been chosen by Goldblatt as the unit for renin (see p. 252 and (24)). However, it is not accepted by all investigators (see p. 253 and (10, 17, 38, 53)).

This method of expression for any substance can be accepted only when it has been demonstrated that injection of unit quantity of the substance causes the same response on repeated injections into the same animal and the same response in a series of different test preparations on successive days. Until such response is demonstrated it is better to compare within a short period in the same animal the response to an unknown preparation with that to a preparation of known strength. Even this procedure may give unreliable results unless there is reasonable proof that the potency of the standard preparation remains satisfactorily constant or that tachyphylaxis does not develop.

Three methods of expression have been used in comparing a standard with an unknown substance: (1) the ratio of response to unit volume of the unknown to response to a similar volume of the known; (2) the ratio of volume of the standard solution to that of the unknown solution which gives an equal response; (3) a plot is made of the dose-response relationship on single log paper to three different amounts of the standard and to three or more injections of the unknown which will produce responses overlapping those of the standard. Unitage of the unknown is then expressed in terms of the linear distance between the two plots along the dosage axis. This procedure reduces the number of injections necessary to obtain an exact match in response and simultaneously allows detection of irregularities in the dose-response relationship which might be due to presence of two oppositely acting substances in the unknown preparation. Whether to use the absolute or percentile change will depend on which remains more constant under control conditions as noted above.

VII. Control of Venous Return

Recent interest has been focused on the mechanisms controlling venous return both under physiologic conditions and in disturbances of homeostasis such as are seen in shock and heart failure. Attempts in this direc-

tion have been made by recording simultaneously the right atrial pressure, cardiac output and aortic pressure. However, interpretations of these data are complicated by fluctuations of central venous pressure produced by respiration, by the fact that extremely large variations in the quantity of blood in the central veins and atria may occur with little change in level of the recorded pressure and, finally, by the fact that cardiac output may vary independently of the central venous reservoir pressure and therefore result in change of atrial pressure in a direction opposite that expected to result from the simultaneously occurring variation in venous return. Fluctuations in capacity of the central venous reservoir and atrium may also cause a change in level of pressure in these structures in the absence of any alteration in cardiac output or venous return, thus further complicating interpretation (26).

Changes in intrinsic capillary and venular tone are probably best estimated from a differential in the rate of change of inflow vs. outflow of a given organ with respect to time, a delayed increase of outflow with respect to increased inflow suggesting dilatation or relaxation of these vessels and acceleration of outflow with respect to inflow suggesting constriction or increase of tone. The magnitude of the storage capacity can probably also be determined from the volume of blood which continues to drain from the venous side after sudden occlusion or cessation of arterial inflow and from the extra volume of arterial inflow which may occur before inflow and outflow become equalized on restoration of circulation.

VIII. Evaluation of Cardiac Contractility

Changes of contractility of the heart may be due (*a*) to alteration of initial length, (*b*) to variation of environment, as by fluctuation of blood temperature, ion content, O_2 tension, hormone concentration or addition of exogenous substances such as digitalis, and (*c*) to change of cardiac nerve activity. Considerable knowledge may be obtained regarding alterations in cardiac contractility from simultaneous measurements of pressure in the right atrium, right ventricle, pulmonary artery, left ventricle† and aorta, intrapleural pressure (12, 76) and cardiac output (see pp. 221 and 224), heart size (44) and velocity of ejection from records of cardiac emptying (75).

Increase in stroke volume in absence of change of intra-atrial, pulmonary artery and aortic pressures provides unequivocal evidence of increased cardiac contractility caused by chemical or nerve influences. Increase in stroke volume or cardiac output in the presence of a rise of atrial or of intraventricular pressure at the beginning isometric contrac-

† Right atrial, right intraventricular and pulmonary arterial pressures may be obtained in man and animals by catheterization via the jugular vein (see p. 224). Left intraventricular pressure may be obtained in the dog by catheterization via the left carotid artery with a woven catheter stiffened during insertion by a metal guide in its lumen (34).

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Cardiac contractility has also been measured directly by Walton and Brodie (see p. 221).

NOTE.—Sections I–VIII were reviewed by Carl J. Wiggers and Irvine H. Page.

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IX. Pressor Effect of Renin and Hypertensin

HARRY GOLDBLATT, *Los Angeles*

Dogs trained to lie quietly on a special support designed for their comfort, on a table and without anesthesia, with only one hindlimb tied down, have their blood pressure taken by the direct method in the limb that is restrained. The ordinary mercury manometer is used with a 20 gauge 1 in. needle inserted through the skin into the femoral artery. To provide the anticoagulant (4% citrate) and to elevate pressure in the manometer to the expected level before the needle is inserted, a pressure bottle with a reducing valve is used. The pressures are usually read without recording them on a kymograph, unless a permanent record is required. As soon as the blood pressure has settled to a fairly fixed level a 23 gauge needle attached to a syringe that contains the test material is inserted in the external saphenous vein of the opposite leg. At a given instant, when the blood pressure is steady, the material is quickly injected and the maximal rise of pressure recorded. Usually the nature of the rise, as well as actual elevation, is noted because from the very nature of the rise one can usually distinguish definitely between renin and hypertensin.

With renin, there is usually a lag of about 15 sec before rise of pressure begins, then the rise is fairly steep, but about 2 min is required for the maximum to be reached. This is usually maintained for several minutes before the beginning of the fall to normal, which may take 30 min or more. With hypertensin, the rise of pressure begins in about 5 sec, is very abrupt, the maximum being reached within $^1/_4$–1 min; then promptly the steep fall begins, and pressure may be back to normal in 2 or 3 min. These are the two pressor-effecting substances with which I have worked for a number of years. Renin is not directly pressor. It is believed to act by way of formation of hypertensin, which is probably the reason for a lag of 15 sec or more before the rise begins. Some investigators do not use this method for the testing of renin. They first produce hypertensin under standard conditions, with the renin, then test for the pressor effect of the hypertensin produced. Page has criticized our method on the basis that the activity of renin, an enzyme, cannot be determined by its direct pressor effect. Nevertheless, in tests of many samples of renin, the potency of which had been determined by other methods, consistent and reliable results have been obtained by our method. Our dog unit of renin is that amount which causes a maximum rise of blood pressure of 30 mm Hg in at least three dogs in 3 min or less. The unit of hypertensin is that amount which causes a maximum rise of 30 mm Hg in at least three dogs within 2 min or less.

For determination of the pressor effect of the intravenous injection of

substances, including renin and hypertensin, others have used the pithed cat. However, these cats must be killed, whereas the dogs can be used for years. For renin determinations, the same dog is rarely used more than once in a single day and never for two successive injections within 2 hr. Sometimes 30 min or longer is required for the blood pressure to return to normal. Several successive determinations of hypertensin, however, can be made on the same animal without tachyphylaxis.

Various methods have been proposed for the determination of blood pressure in the tail of the rat. All the methods are plethysmographic, but most of them are unsatisfactory because they are not entirely objective. A method based on a different principle has been described for determination of the blood pressure in the lower hindlimb of the rat. This seems more satisfactory, but I have had no experience with it.

For study of the vasoconstrictor effect of various substances, including hypertensin, we have used the Läwen-Trendelenburg technique on the giant toad (see p. 129). There are a few pitfalls in the method, but once mastered it is highly satisfactory for at least qualitative determination of the vasoconstrictor effects of substances soluble in water.

X. Standardization of Renin

IRVINE H. PAGE, *Cleveland Clinic Foundation*

The only advantage of methods of renin assay based on the pressor response in intact animals is apparent simplicity. There are at least two fundamental drawbacks: (*a*) renin does not produce the same response in different animals, at least under anesthesia, nor in the same animal at different times in most investigators' hands, and (*b*) repeated injections of renin produce tachyphylaxis, making it impossible to "titrate" renin as is done with such pressor amines as adrenalin which do not produce tachyphylaxis.

Since renin is a catheptic enzyme and not a pressor substance, as it is often carelessly labeled in the literature, logic would require that it be tested for what it is, not for a property of one of its reaction products, angiotonin.

Plentl and Page (1) found the formation and destruction of angiotonin from renin and renin substrate to consist of two consecutive reactions, both of which follow the laws of first order kinetics. The reaction constant is proportional to the enzyme concentration and hence should be used to express the activity of the enzymes. Their method of kinetic analysis may be somewhat more time-consuming but the results, though not perfect, will come far closer to the truth than methods based on the very indirect animal assay. As the requirements for more exact work on

renin increase, arbitrary units based on a physiologic property of a reaction product, will be found to have little exact meaning. An enzyme should be assayed, not as a pressor agent, but for its enzymatic qualities. Added significance might be given physiologic assays if it were shown how they compare with kinetic analysis.

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SECTION III

Selected Methods in Gastroenterologic Research

ASSOCIATE EDITOR—*A. C. Ivy*

INTRODUCTION

THE SUBJECTS chosen for presentation in this section do not form a unified group. In each instance our choice has been guided by three considerations. (1) The topics are ones with which workers in our laboratories have had extensive experience, and we feel that this experience may be a substantial aid to others interested in these fields. (2) The topics are, in our opinion, timely. Each of these fields of research is currently being actively investigated by many groups. (3) Each topic selected presents some methodologic problem. In fact, some of the topics are controversial. It is felt that in these instances much is to be gained by clearly setting forth the practices of our laboratories so that the sources of any discrepancies may be more readily discerned.

—A. C. IVY

ASSAY OF CHOLERETIC COMPOUNDS OR VARIABILITY OF LIVER IN RESPONSE TO STANDARD DOSE OF CHOLERETIC COMPOUNDS

M. J. GUNTER, H. RALSTON *and* A. C. IVY, *University of Illinois*

THE LITERATURE reveals no agreement regarding the best method for assaying compounds for their comparable effect on the volume output of bile by the liver. In fact, agreement does not exist relative to the terminology. The terminology of Brugsch and Horsters (2) is most commonly employed; in this, choleretics are agents which increase the volume output of bile by the liver, and cholagogues are agents which promote expulsion of bile from the gallbladder. Terminology has been discussed by one of us and a rational terminology proposed (6). For this study the term choleretic refers to an increase in bile volume output.

Choice of animal.—Stransky (11) popularized the use of rabbits for assay of choleretic compounds. Rabbits, however, failed to respond to cinchophen, to which dogs and humans respond. Grabe (4) used rats, but Fromherz (3) and Wenner (14) found that rats do not respond as do dogs and humans. So far as is known the human and canine liver respond alike to choleretic substances. It would appear, then, that the dog is the laboratory animal of choice for use in assaying choleretics before they are tried on human subjects.

Animal preparation.—1. *"Chronic biliary fistula" preparation.*—Schwann (10) in 1844 introduced the gallbladder fistula preparation, which has been used as recently as 1936. This is not a good preparation for study of choleretic compounds because the gallbladder mucosa modifies the hepatic bile.

The "chronic fistula" of the common bile duct or of the cystic duct with the common duct tied and sectioned and the gallbladder removed is a good method when used with the precautions introduced by Rous and McMaster (8) and Kocour and Ivy (7).

It is believed that the chronic biliary fistula should be used in the final evaluation of choleretics because use of an anesthetic is avoided and the choleretic is employed under conditions more similar to those under which they will generally be used clinically. The acute biliary fistula, if used properly, is an ideal preparation for selecting compounds to be used in dogs with a chronic fistula. Within eight hours two preparations may be

assayed, whereas much more time and effort are required to make an acceptable assay on the animal having a chronic biliary fistula.

2. *"Acute biliary fistula" preparation.*—This was apparently first described by Rutherford (9) in 1878. The method as we use it consists in the following procedures. (*a*) The dog, having not been fed for 14 hr, is anesthetized with pentobarbital given intravenously. (*b*) The common bile duct is cannulated and the cystic duct clamped with a hemostat (ligating causes unnecessary trauma). (*c*) The cannula and rubber tube (3 mm in diameter inside) are so placed as to prevent mechanical obstruction. A stitch holding the cannula to the side of the duodenum is helpful. The rubber tube is brought to the outside through the abdominal incision or a stab wound, the orifice of the tube being at or slightly below the level of the cannula in the duct. A graduated cylinder is used for collecting the bile. (*d*) While the cannula and tube are filling with the bile being formed continuously, the carotid artery is cannulated and connected to a blood pressure recording apparatus. The blood pressure should be recorded because it affects the formation of bile (12) and a significant change in blood pressure on the injection of a compound provides information pertaining to the toxicity of the compound.

According to our experience, a compound should be injected into at least two dogs before it can be said to have no appreciable choleretic effect. During our work it became necessary to ascertain if the administration of dehydrocholate to a dog in any way influenced the response to a second dose of dehydrocholate or to another choleretic compound after bile flow returned to normal. In 12 dogs the response to the second dose of dehydrocholate was, within the experimental variations, identical with the response to the first dose. Administering potent choleretic compounds to six dogs without the previous administration of dehydrocholate, we obtained responses that agreed closely with those obtained following previous dehydrocholate administration.

The remainder of this discussion deals with the choice of methods for comparing the choleretic action of different compounds in the "acute biliary fistula" dog.

THE ACUTE EXPERIMENT

The acute experiment or actual assay of a compound X is conducted as follows. (*a*) Bile is collected for a *control period* of 30 min. (*b*) Sodium dehydrocholate in a dose of 0.047 mM/kg body weight (20 mg/kg) is injected intravenously over 2 min. (*c*) The bile is collected at 30 min intervals for 1.5–2 hr, at which time the flow has invariably returned to the control rate. The peak rate of flow occurs in the first 30 min after a 30 min control collection, a predetermined amount (*vide infra*) of compound X is injected and the bile collected at 30 min intervals until flow has returned to the control rate.

Theoretical consideration of possibilities of comparing results.—*a*) *Data on control flow during 30 min.*—One hundred dogs, prepared as de-

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2. *"Acute biliary fistula" preparation.*—This was apparently first described by Rutherford (9) in 1878. The method as we use it consists in the following procedures. (*a*) The dog, having not been fed for 14 hr, is anesthetized with pentobarbital given intravenously. (*b*) The common bile duct is cannulated and the cystic duct clamped with a hemostat (ligating causes unnecessary trauma). (*c*) The cannula and rubber tube (3 mm in diameter inside) are so placed as to prevent mechanical obstruction. A stitch holding the cannula to the side of the duodenum is helpful. The rubber tube is brought to the outside through the abdominal incision or a stab wound, the orifice of the tube being at or slightly below the level of the cannula in the duct. A graduated cylinder is used for collecting the bile. (*d*) While the cannula and tube are filling with the bile being formed continuously, the carotid artery is cannulated and connected to a blood pressure recording apparatus. The blood pressure should be recorded because it affects the formation of bile (12) and a significant change in blood pressure on the injection of a compound provides information pertaining to the toxicity of the compound.

According to our experience, a compound should be injected into at least two dogs before it can be said to have no appreciable choleretic effect. During our work it became necessary to ascertain if the administration of dehydrocholate to a dog in any way influenced the response to a second dose of dehydrocholate or to another choleretic compound after bile flow returned to normal. In 12 dogs the response to the second dose of dehydrocholate was, within the experimental variations, identical with the response to the first dose. Administering potent choleretic compounds to six dogs without the previous administration of dehydrocholate, we obtained responses that agreed closely with those obtained following previous dehydrocholate administration.

The remainder of this discussion deals with the choice of methods for comparing the choleretic action of different compounds in the "acute biliary fistula" dog.

THE ACUTE EXPERIMENT

The acute experiment or actual assay of a compound X is conducted as follows. (*a*) Bile is collected for a *control period* of 30 min. (*b*) Sodium dehydrocholate in a dose of 0.047 mM/kg body weight (20 mg/kg) is injected intravenously over 2 min. (*c*) The bile is collected at 30 min intervals for 1.5–2 hr, at which time the flow has invariably returned to the control rate. The peak rate of flow occurs in the first 30 min after a 30 min control collection, a predetermined amount (*vide infra*) of compound X is injected and the bile collected at 30 min intervals until flow has returned to the control rate.

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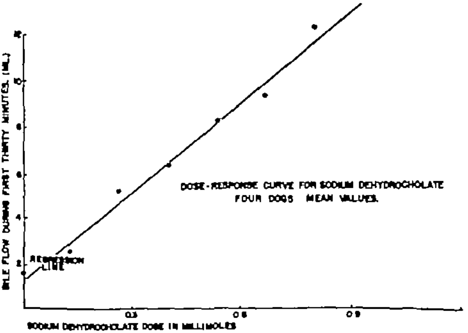

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c) Dose-response curve for dehydrocholate.—In four dogs, a 30 min control flow was obtained, and then 0.14 mM of dehydrocholate, as sodium dehydrocholate (total dose, 60 mg of sodium dehydrocholate), was injected intravenously. When the rate of flow of bile had returned to the control rate (1–2 hr) 0.28, then 0.43, then 0.57, 0.71 and 0.85 mM/kg of

TABLE 2.—RESPONSES OF BILE FLOW OF 4 DOGS TO INCREASING DOSES OF SODIUM DEHYDROCHOLATE

Dose of Dehydrocholate, mM	Mean Responses, ML/30 Min
0.00	1.3
0.14	2.4
0.28	4.8
0.43	6.0
0.57	7.7
0.71	8.8
0.85	11.6

dehydrocholate was administered. Average results are shown in Table 2. The correlation coefficient relating the dose to the response is very high (0.99). The results yield the following regression equation when R stands

scribed, had a mean weight of 8.3 kg, with standard deviation (S.D.) 3.4 kg. The mean control rate of bile flow was 1.10 ml/30 min, S.D., 0.70 ml, and coefficient of variability (100 times S.D. divided by mean), 64.

b) *Expression of secretory response.*—There are three ways to express the secretory response to an excitatory substance such as sodium dehydrocholate, which was the standard excitant in this study: (1) calculate the percentage increase in volume output over that of the control; (2) use the actual increase in milliliters of bile over that of the control; (3) ignore the control flow and use the amount in milliliters of bile collected after the injection.

In determining which of the foregoing criteria is the least variable, it was necessary to determine first the length of the period of bile collection the volume of which is to be used. The volume of the first 30 min or the first hour after the injection might be used.

In 40 dogs, bile output following administration of 0.047 mM of sodium dehydrocholate was determined at 30 and at 60 min. Mean output during the 30 min was 6.5 ml (S.D., 2.4 ml; coefficient of variability, 36.4); mean output during the 60 min was 9.4 ml (S.D., 3.6 ml; coefficient of variability, 38.4). Hence the mean output during the second 30 min was 2.9 ml.

It is apparent that the maximal effect of dehydrocholate is exerted during the first 30 min following administration, and that during the second 30 min the rate of secretion is well on its way back to the control rate of flow. The variability of the 30 min collection is no more than the variability of the 60 min readings. This is true of all of 80 compounds assayed for choleretic action to date. The maximal effect always was exerted during the first 30 min following intravenous administration.

TABLE 1.—Three Ways of Expressing Increase in Bile Flow in Response to Sodium Dehydrocholate

Response	Mean	Standard Deviation	Coefficient of Variability
% increase over control	770%	646%	83.9
Absolute increase over control	4.74 ml	2.02 ml	42.6
Absolute response flow	5.79 ml	2.30 ml	39.7

Table 1 shows data which demonstrate that the volume output during the first 30 min after injection of dehydrocholate has the lowest coefficient of variability. This is due to the large variations in volume output during the control period. The percentage increase over the control flow is twice as variable as the actual volume of bile collected the first 30 min after the injection of dehydrocholate.

Comment: Since the bile flow in response to dehydrocholate is subject to less variation than the control rate of flow, it follows that the choleretic potency of a drug can be evaluated more reliably by comparing the response to the drug to be tested with the response to dehydrocholate than by comparing the effect of the drug with the control rate of flow. There-

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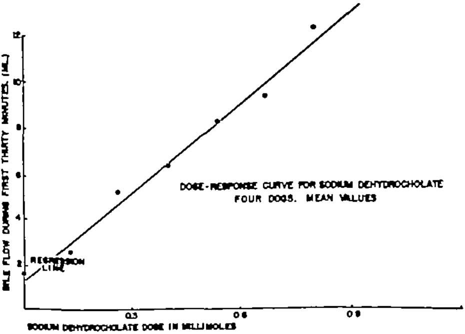

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d) *Use of regression equation or curve.*—If D is the equivalent dose of dehydrocholate, the relative potency, P, of a compound can be calculated by dividing D by the dose of the compound used, D', and multiplying by 100, or

$$P = \frac{D}{D'} \times 100.$$

If the potency of the compound is equal to that of dehydrocholate, P will equal 100. The greater the value of P, the greater the potency of the compound.

Example: One millimole of compound A produces 5 ml of bile during the first 30 min after its injection. The equivalent dose of dehydrocholate, D, is obtained from the regression equation: $R = 1.044\ (12.0 \times D)$, when R equals 5.0 ml and D, 0.33 mM. Thus, 1 mM of compound A is equivalent to 0.33 mM of dehydrocholate. Hence, the relative potency of compound A will be

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or, combining the potency and regression equations, since

$$D = \frac{R - 1.04}{12.0},$$

$$P = \frac{100\ (R - 1.04)}{12.0 \times D'},$$

and if $D' = 1.0$ millimole and $R = 5$ ml, then

$$P = \frac{100\ (5 - 1.04)}{12.0 \times 1.0} = 8.35 \times 3.96 = 33\%.$$

The value can be read from Figure 1. For example, if 1 mM/kg produced 5 ml of bile in 30 min, it will be found from Figure 1 that 5 ml of bile is produced by 0.33 mM of dehydrocholate.

TABLE 3

Compound	No. of Dogs Used in Assay	Relative Potency Compared to Dehydrocholate	Stand. Dev.	Coefficient of Variability
1	6	55	24	44
2	8	53	14	26
3	7	52	10	37
4	6	50	21	42
5	7	46	13	28
6	8	43	17	40
7	7	31	14	45
8	6	23	10	44

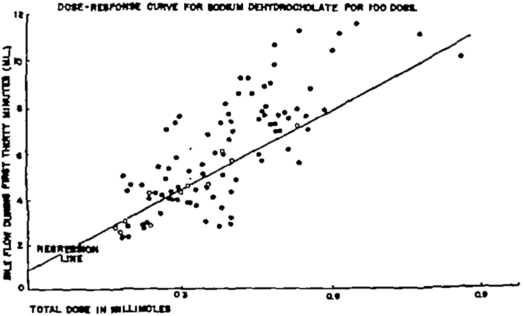
Note.—Recently we have used new synthetic compounds which after a single injection intravenously stimulate bile volume output for 3 and 4 hr. This is a special problem that will be discussed elsewhere.

for the bile volume output during the first 30 min after injection, and D, the dose of dehydrocholate in millimoles ($12.0 \times D$),

$$R = 1.04 + (12.0 \times D).$$

The graph of the equation and the results are shown in Figure 1.

The dose-response was then constructed for the 100 dogs, previously referred to, which received a fixed dose of dehydrocholate per kilogram of body weight (0.047 mM/kg). Using the control rate of flow which corresponds to an exogenous dose of 0 mM, it was possible to construct a curve based on 200 observations on bile flow. In addition, the record of



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total dose each dog received could be used for constructing the graph and equation. The correlation coefficient relating dose of dehydrocholate to volume output of bile during the first 30 min is +0.62. The coefficient is less than for the four dogs because of the variability of the control flow, or presumably the amount of endogenous bile acids acting at the time of injection of the exogenous bile acid; the coefficient is less also because the 100 dogs received only one dose of dehydrocholate (Fig. 2), whereas each of the four dogs received several increasing doses. In fact, Figure 2 is shown only because of the remarkable correspondence of the two regression lines.

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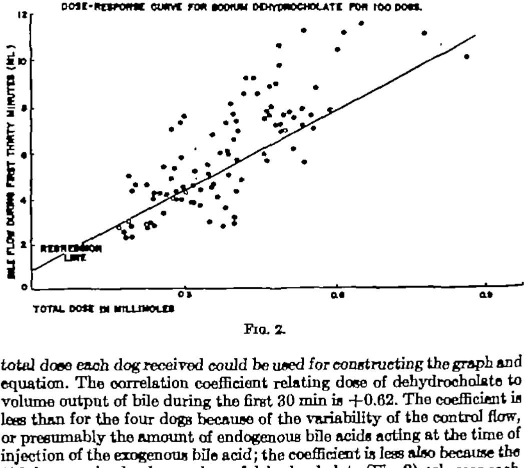

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PREPARATION AND USE OF THE MANN-WILLIAMSON DOG

M. I. GROSSMAN and A. C. IVY, University of Illinois

Of the many methods proposed for experimental production of peptic ulcer, only a few have proved to be of value for routine use. Because of its reliability and the vast knowledge gained about it, the Mann-Williamson dog (4) stands first among the experimental methods for ulcer production. The M-W dog presents certain definite disadvantages for use in assessing therapy, particularly the long period of observation required. However, it is the most reliable method available, and until a better one is provided it should be used.

PROCEDURE

Operative procedure.—Male and female mongrel dogs weighing between 6 and 15 kg are used. They are placed in cages and observed for at least 1 week before operation in order to detect and eliminate any animals with obvious abnormalities such as distemper, worms and malnutrition. The dog is starved for 24 hr before surgery. Open drop ether anesthesia is used after premedication with morphine sulfate (2 mg/kg) and atropine sulfate (0.15 mg/kg) given subcutaneously $^1/_2$ hr before anesthesia is started.

Through a right rectus muscle-splitting incision the hepatoduodenal ligament is cut to mobilize the pyloric sphincter region (Fig. 1). The right gastric and right gastroepiploic vessels are doubly ligated and transected opposite the pyloric sphincter. A rubber-covered intestinal clamp is placed on the stomach several inches above the pyloric sphincter and an Allis forceps is placed on the superior border of the duodenal bulb. The branches of the right gastroepiploic vessels running from the cut ends of the vessels to the duodenal wall are ligated with a suture ligature and the pyloric sphincter is then transected. The duodenal mucosa is infolded by sliding the muscularis and serosa over it, and the duodenal stump is closed by two continuous rows of Lembert sutures through the serosa and muscularis.

The first loop of jejunum is now identified and the arched mesenteric vessels parallel to the intestinal border are doubly ligated and transected. The jejunum is transected at the point where the vessels have been

Critique.—The foregoing method was used in the assay of eight compounds with choleretic action. Like sodium dehydrocholate in the dose used, they all stimulated the formation of bile in every dog to which they were given intravenously. The large variability of choleretic response in different dogs is clearly evident from the results (Table 3). This is also true of the response to sodium dehydrocholate, since as cited above, the response to a standard dose (20 mg/kg) in 100 dogs was 5.79 ± 2.3 (S.D.), the coefficient of variability being 40.

The liver is no exception among the digestive glands regarding variability in response to a standard secretory stimulus. The variability in response of the salivary glands to the same dose of pilocarpine (1), of the gastric glands to the same dose of histamine (13) and of the pancreas to the same dose of secretin (5) is of the same order of magnitude as that of the liver.

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If the dog is to be treated with medicines given orally or parenterally, treatment is begun one week postoperatively.

Diet.—The basal diet fed all our Mann-Williamson dogs postoperatively, including both control and treated groups, consists of: commercial dog food (Pard, Swift), 1 can; fresh ground raw hog pancreas, 100 g; fresh ground raw hog liver, 100 g; fresh whole milk, 200 ml.

The M-W dog manifests considerable food wastage as is seen by the bulky, soft, light-colored stools containing appreciable quantities of undigested food. It is therefore necessary to supply this diet in liberal quantities to maintain the animal's nutritional state. When this diet is used instead of the usual kennel ration, average postoperative survival of M-W dogs is distinctly prolonged. Thus the average postoperative survival time of 114 control animals receiving this diet was 110 days. Other laboratories using an ordinary diet report lower average survival times; for example, Sandweiss' group (1) had an average postoperative survival time of 72 days for 29 control M-W dogs.

Incidence and time of occurrence of ulcer.—Among untreated animals ulcers develop in all except two groups. (1) The first consists of those which do not live long enough to develop an ulcer. Since average time of onset of ulcer is about 7 weeks, if death due to extraneous causes occurs early postoperatively, there may not have been time for development of ulcer. (2) About 2 per cent of animals fail to develop ulcer, even without special treatment other than the diet. These animals may live indefinitely. When an animal does not develop ulcer within 1 year after operation, ulcer is unlikely to develop later. On the other hand, if ulcer has been prevented from occurring for a year or longer by treating the animal, for example with aluminum phosphate, ulcer will occur in the usual length of time after treatment is discontinued. In other words, prevention of the ulcer for a year does not produce an adaptation which prevents ulcer from occurring when treatment is stopped.

The commonest error made in evaluating the results of a certain treatment in M-W dogs is to consider that animals which die without ulcer within a few weeks after operation have been benefited by treatment. When death without ulcer occurs within a few weeks after operation it is not possible to determine whether the absence of ulcer is due to lack of sufficient time for ulcer to develop or to the beneficial effect of therapy.

Since diet and other variable factors are so important in modifying the course of the M-W dog, it is essential that each laboratory do its own control series of animals. The control series should be done concurrently with the treated animals, and the same team of surgeons should perform the operations.

Figure 2 depicts the postoperative survival times of 114 control (untreated) M-W dogs. At the end of 12 weeks 50 per cent of the animals had died with ulcer. Dogs surviving less than 4 weeks were excluded (see p. 267). Average survival time for the entire group was 15.2 ± 0.7 weeks, excluding two animals which survived more than 90 weeks without de-

ligated and the distal end of the jejunum is closed by two continuous rows of Lembert sutures through the serosa and muscularis.

The pyloric end of the stomach is now anastomosed to the jejunum about 1 in. distal to the closure that has just been made. The first continuous row of sutures joins the serosal border of the posterior surface of the cut end of the stomach to the serosal surface of jejunum near the mesenteric border. *The jejunum is opened to produce a stoma of* approximately the same size as and opposite that of the stomach. An inner continuous row of Connell sutures completes the posterior half of the anastomosis. The Connell suture is continued to join the anterior mucosal layer of the stomach and the other lip of the jejunum. A con-

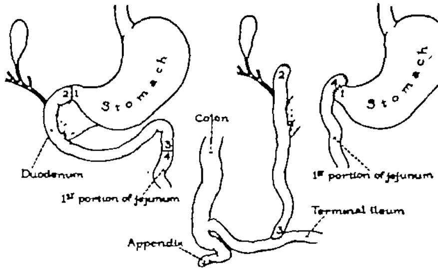

FIG. 1.—Procedure for Mann-Williamson operation.

tinuous row of Lembert sutures joining the anterior serosal edge of the stomach to the serosa of the jejunum completes the anastomosis.

The continuous suture lines should be interrupted at several points by *tying a knot and restarting in order to avoid* excessive "purse-stringing" and narrowing of the anastomotic stoma. The small segment of jejunum above the anastomosis is sutured to the gastric wall to prevent intussusception. The terminal ileum is now identified and mobilized. The upper end of the cut jejunum is anastomosed end-to-side to the terminal ileum 15 cm (accurately measured) above the ileocecal junction. The suturing technique is similar to that used in the gastrojejunal anastomosis.

Fine cutting needles and ordinary white mending silk are used throughout.

The abdomen is closed in layers and an apron binder or a collodion dressing is applied.

Food and water are withheld for 3 days postoperatively during which time physiologic saline solution (50 ml/kg) is given subcutaneously once daily.

diet employed by Shoch and Fogelson (6). Therefore the basic criterion for evaluation of therapies is the average postoperative survival time. In using this measurement, standard statistical methods should be applied to determine whether the difference between average survival times of the treated and untreated groups is significant. In Table 1 two examples of such a calculation are given.

TABLE 1

Treatment	No. of Dogs	Mean Postop. Surviv., Days	Sum of Squares	Diff. of Means, Days	t	p
Example 1 (5)						
None, control	29	72	23,535	29	0.58	>0.05†
Histidine	10	101	55,625			
Example 2 (6)						
Casein diet	10	131	82,202	92	2.04	0.05*
Hydrolyzed casein diet	15	223	171,693			

$t = \bar{x}\sqrt{\frac{n_1 n_2(n_1 + n_2 - 2)}{(n_1 + n_2)\Sigma x^2}}$ [see Snedecor (7)]

$\bar{x}$ = difference of group means.

n_1, n_2 = number of animals in each group.

Σx^2 = pooled sum of squares of deviations from means.

p = p is read from a table in which t and n are the variables. When p is 0.05 or less, the difference is considered significant; i.e., it is concluded that the probability of such a difference arising by chance due to random sampling error is less than 5 in 100.

† = not statistically significant.

* = statistically significant.

Using these same statistical formulae it can be calculated that 10 dogs would have to manifest an average longevity of approximately 20 weeks (or 4.8 weeks longer than the untreated controls) to produce a significant difference from our control group (see Fig. 2). This calculation is based on the assumption that the degree of variation in the treated group would be of the same order of magnitude as that experienced in the control group. Expressed in another way, if 50 per cent of 10 treated animals were still alive at the end of 16–17 weeks we could conclude that the therapy had been beneficial. It should be emphasized that these hypothetical calculations hold only for comparisons made with our control group of animals

The statistical method described here is applicable only when the frequency-distribution curve of incidence of mortality during successive time periods does not differ significantly from normal. When distribution is other than normal, such as is encountered when treatment is strikingly beneficial and prevents ulcers for many years in some dogs, then more complicated methods are required, such as the time-mortality analysis of Bliss (1a).

In calculating the mean postoperative survival time it is our practice to eliminate all dogs which have not survived a certain arbitrarily predetermined minimal survival time. This applies to both control and treated groups. We have chosen 30 days as the most suitable time for this pur-

veloping ulcer. Again excluding those two animals, all had died with ulcer by the fortieth postoperative week.

Criteria for evaluation of therapy.—When therapy is strikingly beneficial no special analysis of the data is required to establish the significance of the results. For example, with enterogastrone (3), urine extracts (1) and aluminum phosphate (2) therapy the average survival time was so much greater than in the control group that the value of these treatments was obvious. However, at times it becomes necessary to decide whether a therapy which does not produce such striking results has had any beneficial effect or whether the difference between control and treated groups is due merely to chance variation. We then are faced with a problem in statistics and must select some objectively measurable

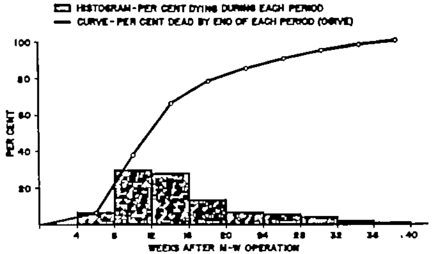

FIG. 2.—Time of death from ulcer in 112 Mann-Williamson dogs untreated except for a diet containing raw pancreas and liver. These data do not include two animals which survived more than 90 weeks; thus two (or 1.8 per cent of 114 animals) did not develop ulcer in almost 2 yr.

criterion for making the statistical comparison between the control and treated groups.

Three criteria have been suggested for use in evaluating therapies used in the experimental M-W ulcer: (1) postoperative survival time (most extensively used); (2) percentage incidence of ulcer in the control and treated groups, and (3) state of the ulcer when present, including observations on tendency to heal or tendency to perforate.

The question now arises: Which of these criteria (or combination of criteria) will give the best means of scoring the efficacy of various therapies? One fact is of dominant importance in making this choice, namely, that some therapies may be only mildly beneficial, delaying onset and progress of the ulcer but not preventing it. In these cases the incidence of ulcer will not be different in the control and treated groups but average postoperative survival of the treated animals will be significantly different. A good example of a therapy of this type is the casein hydrolysate

diet employed by Shoch and Fogelson (6). Therefore the basic criterion for evaluation of therapies is the average postoperative survival time. In using this measurement, standard statistical methods should be applied to determine whether the difference between average survival times of the treated and untreated groups is significant. In Table 1 two examples of such a calculation are given.

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In calculating the mean postoperative survival time it is our practice to eliminate all dogs which have not survived a certain arbitrarily predetermined minimal survival time. This applies to both control and treated groups. We have chosen 30 days as the most suitable time for this pur-

pose. We do not count animals which have failed to survive this minimal period because during this period animals frequently die, with or without ulcer, from complications of the surgical procedure. Therefore death during this period is frequently not related to the ulcer development. Pneumonia and intestinal obstruction are common causes of death during this period. The extraneous causes may end the life of the animal before an ulcer has had time to develop. It is a common error to consider animals which have died within a few weeks of the M-W operation without ulcer to have been protected by some therapy they happened to be receiving at the time. This, for example, was the origin of the histidine fad in the treatment of ulcer. The minimal requirement that any therapy must fulfil in order to be considered beneficial is that it produce a significant increase in the postoperative survival time.

The percentage incidence of ulcer (corrected for minimal survival time) may be used as an additional criterion of efficacy when it is applicable. If two therapies result in the same mean postoperative survival time but the incidence of ulcer in one group is significantly lower than the other, then the group with lower ulcer incidence may be considered to have been more greatly benefited. However, statistical concepts must be applied here again, and in this situation the chi-square test (7) is the most suitable device for testing the significance of the difference in incidence of ulcer in the two groups. Practically, such a situation will rarely be met because therapies which decrease the incidence of ulcer will prolong the life of the animal. No accurate means of combining postoperative survival and ulcer incidence is available; the first is a measurement datum, the second an attribute datum. It is therefore recommended that ulcer incidence be used as a separate, adjunctive criterion.

The healing tendency of the ulcer has been used as a criterion of the value of therapy by Sandweiss and co-workers (1). It presents difficulties because it cannot be measured objectively with accuracy. It is probably of value as an additional criterion.

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STUDY OF GASTRIC ACIDITY IN MAN

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Systematic determinations of the acidity of the gastric contents are usually made to investigate normal physiologic processes, to compare the findings in certain diseases with the normal values and to evaluate the effect of various drugs and factors on gastric secretion and the acidity of the gastric contents.

General considerations.—Hydrogen ion determinations are best made on aspirated gastric juice. Use of the glass electrode at the tip of a stomach tube has been advocated for determination of pH in situ. This is not recommended because of uncertainty as to the position of the glass bulb. Although the exact position of the tip of an aspirating tube is not known either, in removing a fluid sample, error due to differences in pH of gastric contents at different regions is minimized owing to mixing.

Since gastric secretion is subject normally to wide spontaneous fluctuations and many physiologic stimulatory and inhibitory influences, strict attention to details is required in the design of an experimental study.

To insure statistically meaningful results the number of cases must be large if small differences are being evaluated. Although small differences may thereby be shown to be statistically significant, it is to be remembered that they may not necessarily be of physiologic or pathologic importance.

The Rehfuss type tube is preferred ordinarily, since the weighted tip simplifies complete emptying by changing the patient's position. Expectoration of saliva is advised to prevent swallowing and admixture with gastric contents. Gastric juice specimens should be filtered through gauze when large particles are present. Samples are usually titrated immediately to "prevent loss of acid," but data have not been found to show that there is a change in pH of a filtered specimen on standing for several hours in the absence of large quantities of mucus.

Töpfer's reagent and phenolphthalein are satisfactory indicators for titration of routine clinical specimens. In the case of Töpfer's reagent, however, the change from red to yellow occurs over such a large pH range (2.0–4.0) that it is difficult to obtain consistent results at the proper level. When using phenolphthalein, with which the change from colorless to pink may occur between pH 8.3 and 10, erroneously high levels of "combined acid" may be reported. For more accurate results

Hollander and Penner (6) recommended the use of bromphenol blue and phenol red as indicators.

Some investigators discard samples containing bile. This results in the waste of many specimens and, in our experience, is not necessary if one is studying the acidity of the gastric *contents* rather than that of pure secretion. In many instances it would be difficult to decide whether or not there was enough "bile tinge" to indicate a significant amount of enterogastric regurgitation. The presence of bile in the specimen, however, should always be recorded.

Study of basal secretion.—This is difficult, as evidenced by the many conflicting reports. Since the volume of secretion is small, incomplete aspiration may result in a relatively large error. Particularly in studying nocturnal secretion, when one does not wish to awaken the subject to change his position, such errors may be large.

Many reports on basal secretion have been based on aspirations at intervals of 1 hr or more. It is not possible in results obtained by this method to differentiate hypersecretion from retention. Hence to study acid output it is necessary *to employ continuous gastric aspiration* or complete emptying at no longer than 10 or 15 min intervals. Mechanical continuous suction devices are unreliable unless under constant observation. We have not found that continuous aspiration provides more accurate results than emptying at 10 or 15 min periods.

One of the most important criticisms of work published on basal secretion has been selection of subjects. Those designated as "normals" should have no evidence of either organic or functional disease affecting the gastrointestinal tract. Ideal controls should resemble the experimental group as closely as possible except for the condition or disease under study. In the case of patients having peptic ulcer, the groups should be in the same status at the time of testing, i.e., in regard chiefly to pain, presence of an ulcer niche and prior therapy.

Finally, one must be certain that the secretion obtained is actually basal. A *neutral environment is provided for the tests*, avoiding cephalic or "emotogenic" secretory stimulation. Fasting for at least 10 or 12 hr is required to be certain that the intestinal phase of gastric secretion has subsided entirely. There is no evidence that presence of the gastric tube has an important effect on secretion.

Test meals.—Those consisting of food or food extracts have been used widely. The *Ewald type* is composed of bread, cookies or similar cereal product, with water or tea. Using this test Vanzant and her associates (12, 13) have analyzed several thousand cases and obtained important information on gastric secretion in normal persons and subjects with various diseases. Because the secretory response is so variable in this procedure its research use is necessarily limited to large series of patients.

To provide a stronger physiologic stimulus, *meat extracts* have been employed in aqueous solution. This technique has been improved by

Wilhelmj *et al.* (14) to include phenol red for the purpose of studying gastric emptying and duodenal regurgitation into the stomach. Occasionally by this method it has been calculated that hypertonic concentrations of hydrochloric acid were secreted. This result has been attributed to water absorption, the test meal being hypotonic (1, 10, 14). It has been recommended, therefore, that the test meal be of isotonic composition (1). Although the Wilhelmj procedure is of value for research purposes, it offers no clinical information which cannot be obtained by simpler methods.

With these and other food test meals, information on emptying time can be obtained satisfactorily. As to the effect on gastric acidity, however, results tend to be obscured because of variability in the cephalic phase of secretion and in the buffering effect of the food substances.

Alcohol has been used extensively for experimental stimulation of gastric secretion, usually to compare the response in normal subjects with that in patients having peptic ulcer. For this purpose the caffeine test meal is reported to be superior, showing distinct qualitative as well as quantitative differences (11).

The technique for the *caffeine test meal* (11) includes the precautions described above for gastric analyses. Following collection of basal secretion for three 10 min periods, 200 ml of warm water containing 0.5 g of caffeine with sodium benzoate is injected into the stomach through the tube. Thirty minutes later the gastric contents are emptied and discarded after a sample has been titrated. Thereafter complete aspirations are made at 10 min intervals for 90 min or until the basal level is reached again.

The secretory response to histamine, a maximal stimulus, is more constant than that to either alcohol or caffeine. For studies involving repeated tests on the same subjects this property is most valuable.

The *histamine test* which we use is modified from that of Bloomfield and Polland (3). After collection of basal samples, histamine in the dose of 0.01 mg/kg of body weight is injected subcutaneously. The stomach is emptied at 10 or 15 min intervals, usually for 90 min.

The *insulin test* has been applied by Holländer (5) to study of patients subjected to section of the vagus nerves. Insulin-induced hypoglycemia apparently requires the presence of vagal fibers to the stomach to stimulate acid secretion. A positive response to the test therefore indicates that vagal innervation to the stomach has not been completely interrupted. On the other hand, a negative response may result not only from complete vagus section but from inadequate or excessive drop of blood sugar levels or transitory refractoriness for unknown reasons. To be sure of the meaning of a negative result it is thus necessary to perform repeated tests, each with blood sugar determinations.

To study acid secretory response quantitatively, as in the case of basal secretion, continuous or frequent complete emptying is required.

When a fractional* procedure is used, removing 5–10 ml at each period, the data are incomplete because only acid concentration can be calculated, instead of the more important figure for total output. Unless complete aspirations are made, additional error results from emptying through the pylorus.

Effects of drugs.—The effects of drugs may be examined from two standpoints, the influence on the quantity of acid secreted, or that on the acidity of the gastric contents.

In study of parenteral secretory stimulants or depressants a complete aspiration technique such as that described earlier is required. In study of the continued effect of drugs acting within the stomach, such as antacids, withdrawal of small samples is advisable.

Since the chief use of antacids is in the treatment of peptic ulcer, usually alternating with milk and cream, the most direct method for evaluation of such drugs is study of their action as they are intended to be used clinically. One such technique, used extensively by Kirsner and Palmer (7–9), consists of hourly administration of the antacid, with 90 ml of milk and cream between doses of the drug. Samples are aspirated immediately before each dose of antacid and of milk and cream.

Primarily for purposes of comparison with similar drugs, antacids may be studied for their effect on gastric acidity induced by stimulatory agents. For this purpose histamine may be preferred because of the greater uniformity of secretory response in given subjects (3). Evaluation of results thus can be made by comparisons between acid levels when the antacids are used and control levels.

This method may be modified by using the block† method (2), in which the gastric contents are emptied at each period, the volume measured and all but a 10 ml aliquot returned to the stomach through the tube. Such a technique offers more complete information than any other, enabling approximate estimation of the total amount of acid secreted as well as effect of an antacid, a test meal or a drug on gastric emptying.

It is critical in the study of neutralization by antacids when using strong secretory stimulating drugs that the dose of antacid be carefully considered. If the dose of antacid is too small or too large compared to acid output, differences between various drugs in regard to buffering ability may not be observed. Use of a large volume of antacid is preferred to decrease the proportion of this drug lost when samples are withdrawn.

Units.—As Hollander (4) has clearly explained, the terms "clinical units" and "degrees of acidity" should be discarded. It is most useful to describe *amounts* of hydrochloric acid as milligrams or milliequivalents

* The term "fractional," introduced by Rehfuss, applies to any analysis procedure in which small samples of stomach contents are aspirated frequently.

† The term "block" describes any analysis in which there is frequent complete emptying and re-injection of the material, retaining a small sample.

and *concentration* as milligrams per milliliter or milliequivalents per liter. Since milliequivalents per liter is numerically equal to "clinical units," conversion offers no difficulty.

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SECTION IV

Cellular Respiration

ASSOCIATE EDITOR—*Van R. Potter*

INTRODUCTION

THE STUDY OF cellular respiration has as its goal the collection of data that will lead to an understanding of the physiology of the organism as a whole in terms of its components. In recent years investigators have become impressed with the need of correlating the events that occur at one level of organization with those that occur at higher and lower levels of organization. The various methods included in this section are not alternative approaches: each method provides data that can supplement the information obtained by the other methods and which, in turn, must be supplemented by them. Thus the homogenate technique yields information as to the concentration of specific enzymes in tissues and is also useful as a basis for study of the effect of various inhibitors on single enzymes. The analysis of tissues frozen in vivo provides some insight into the balance between the individual enzymes in the living animal, while the slice technique measures the over-all respiration of the tissue sample. The measurement of respiration of the intact animal in turn reflects the sum of all the component tissues and may reveal metabolic disturbances that occur in tissues in general.

A fifth approach is the study of single organs by means of the Fick principle (pp. 191–217, 224 ff.). By simultaneous measurement of the rate of blood flow and of the amount of oxygen (or metabolite) in the arterial and venous blood of the organ, the rate of metabolism can be obtained.

Few physiologic conditions have been studied from all five approaches. It is relatively easy to demonstrate effects of inhibitors on enzyme systems, but the mere demonstration that a given enzyme can be inhibited does not mean that this fact is of physiologic importance, since the physiologic effects may be caused by the interference with an entirely different and more sensitive enzyme.

An example of a compound that has been studied at several levels of organization is hydrocyanic acid. The lethal action of this compound is well known, and studies with tissue slices have shown that it prevents the uptake of oxygen. Studies with tissue homogenates show that cytochrome oxidase is inhibited at cyanide concentrations that have essentially no effect on other enzyme systems. The effect of cyanide has been studied in the case of intact animals by Dr. Robbie, and its effect on the intact brain has been studied by the methods of freezing in situ by Olsen and Klein (Olsen, N. S., and Klein, J. R.: J. Biol. Chem. 167:739, 1947).

In their presentation of the techniques in the four sections of this chapter, each author has followed his own interpretation of the goals set forth by the Governing Board. The section on the respiration of small animals represents an extension of the use of the Warburg apparatus and *is noteworthy because it employs the standard Warburg manometer to* measure the rate of oxygen uptake in contrast to the scores of devices that have been built according to individual design. It would obviously not have been feasible for Dr. Robbie to discuss all of the devices that have been employed since the time of Atwater for the measurement of oxygen consumption by intact animals. On the other hand, Dr. Field was able to cite a large number of relevant papers, all concerned with measurements with a standard piece of apparatus, the Warburg respirometer.

Although the goal of having each method repeated by a second investigator is highly desirable, it is a good deal less feasible for a method of study than it is for a method of synthesis or preparation, because the criteria of excellence are much less objective and judgment is more likely to involve opinion. However, we have been particularly fortunate in securing the co-operation of Dr. W. E. Stone and Dr. H. G. Wood for comments on the in situ freezing technique and on the homogenate technique, respectively.

—Van R. Potter

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A fifth approach is the study of single organs by means of the Fick principle (pp. 191–217, 224 ff.). By simultaneous measurement of the rate of blood flow and of the amount of oxygen (or metabolite) in the arterial and venous blood of the organ, the rate of metabolism can be obtained.

Few physiologic conditions have been studied from all five approaches. It is relatively easy to demonstrate effects of inhibitors on enzyme systems, but the mere demonstration that a given enzyme can be inhibited does not mean that this fact is of physiologic importance, since the physiologic effects may be caused by the interference with an entirely different and more sensitive enzyme.

Warburg manometer at the end of the stopcock. The connection usually used for a Warburg flask is closed off with a cap made by cementing a piece of rubber stopper into a short piece of large rubber tubing. The second and third chambers are interconnected, and a lead from them goes to another Warburg manometer tube. The pump continuously circulates air through this part of the system.

Pump.—The pump consists of a $^{4}/_{16}$ × 22 in. piece of soft gum rubber tubing coiled once inside a cylindrical hole in a wooden block, with each

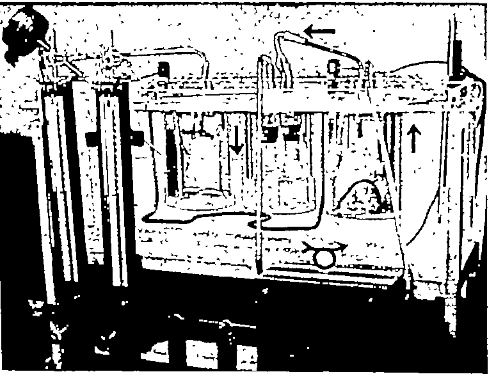

FIG. 1.—Constant flow respirometer used in measuring O_2 consumption of a white rat. The rubber tube pump continuously circulates air through the chamber containing the animal and through the adjoining one in which CO_2 is removed. The left-hand, empty chamber acts as thermobarometer.

end passing through the block to attach to glass connecting tubes (Fig. 2). Air is driven through the rubber tubing by a free-moving wooden roller which pushes the gas ahead of it as it squeezes the rubber. This roller is driven by friction contact with another roller of about the same size which is coupled to the drive shaft of a reducing gear on an electric motor. Ball bearings for the main shaft are mounted in the detachable wooden front and back plates. If a good quality of tubing is used and the rollers are well fitted so that compression is no more than that necessary to drive the air, the rubber will last a long time. It can be checked for leaks by clamping one end and forcing air in at the other end with a glass syringe while the tubing is under water. The pump with dimensions

MEASUREMENT OF RESPIRATION OF INTACT ANIMALS WITH THE CONSTANT FLOW RESPIROMETER

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MEASUREMENT OF OXYGEN consumption in the conventional Warburg manometer is ordinarily limited to materials which are sufficiently aerated by shaking of the liquid medium. The constant flow respirometer retains the advantages of the manometric principle and still allows observations of respiration of intact animals whose size exceeds the capacity of the direct Warburg system. This is accomplished by aerating the system with a continuously flowing stream of gas rather than depending on shaking to obtain oxygen equilibrium. The respirometer is essentially a constant volume, Warburg-type manometer equipped with a pump to circulate air continuously (6, 7). Since the chambers are submerged in a thermoregulated water bath and a thermobarometer is present to correct for changes in temperature or pressure, the calculation of results is as simple as with the ordinary Warburg technique. Sensitivity of the system is sufficient to permit observations of oxygen consumption at frequent intervals, and in this way it is possible to obtain a continuous record of the respiration of the animal being studied.

The importance of correlating studies of isolated tissues and enzyme systems with measurements on intact animals is obvious, and the method provides a convenient and sensitive way of carrying out the experiments on whole organisms. It is useful also for basal metabolism and respiratory quotient determinations, for observations on aquatic forms and for experiments on the effects of drugs and anesthetics on tissue respiration.

The following pages present construction of the apparatus and use of the method in measurements of the respiration of small mammals, aquatic animals and certain types of isolated tissues that are disrupted by shaking.

APPARATUS

Figure 1 shows equipment designed for measurements of respiration of small laboratory animals such as white rats. It consists of a rubber tube pump, three plastic chambers immersed in a transparent, plexiglas, constant temperature water bath (8) and two Warburg manometer tubes. The first chamber acts as a thermobarometer and is attached to a

All gas connections with the chambers are made through holes in the top plate by short pieces of plastic tubing cemented to the plate.

The top plate is equipped with lugs on both sides; by inverting the plate and using a set of chambers $2^1/_2$ in. in diameter and 4 in. deep, sensitivity is increased about six times. With the small containers the respiration of mice or infant rats can be measured as precisely as that of larger animals in the big chambers.

Since several connections are made with rubber tubing, the equipment should be tested occasionally for leaks. This may be done by setting it up without respiring material and comparing the readings with those of the thermobarometer. If tight-fitting connections are made there is seldom any difficulty.

After the chambers are prepared and the animal is in position the top rings are covered with a sealing material,* the chambers are twisted into the locked position, and the plastic plate with the three attached containers is set into place in the water bath. Tubes from the pump are connected and the air flow started.

Air moves from the pump to the third, or right-hand, chamber, which contains the animal. It is led out through a glass tube that is extended to the bottom of the container to prevent accumulation of CO_2, then passes in a glass tubing bridge to the middle chamber. The air here bubbles through alkali which removes the CO_2, then returns to the pump and repeats the circuit. A lead from the top of the third chamber goes to a manometer tube, and change of pressure within the system is indicated by a change in the column of manometer fluid.

Aquatic animal respiration measurements.—The same apparatus may be used for measuring respiration of aquatic animals, because of the limited solubility of oxygen in water.† As oxygen is removed from the water by the animals it is replaced by the supply in the air, and the resulting drop in gas pressure is measured manometrically. It is necessary only to remove the CO_2 which is produced and to bubble the air through the water in the chamber containing the animal at a rate rapid enough to maintain equilibrium between the free and the dissolved gas. Since the rate of respiration of cold-blooded animals is usually low, the rate of circulation employed in the apparatus described for use with rats is ordinarily sufficient. Carbon dioxide is largely retained in sea water if the buffering capacity is not overloaded, but the simplest way to remove the gas is to use tubes of alkali in the second chamber.

Micromodification.—The principle of the constant flow respirometer is not limited in application to measurement of large volumes of gas exchange. Figure 3 shows a series of small respirometers with sensitivity equivalent to that of the conventional Warburg manometers with

* Sealing mixture is made up by warming equal parts of petroleum jelly and anhydrous lanolin with enough liquid petrolatum to form a smooth-flowing seal.

† One hundred volumes of water in equilibrium with air at 15 C contains only 0.72 volume of oxygen (2).

specified in the figure delivers about 250 ml of air/min. If more circulation is required, several lengths of tubing may be fitted into the same block and interconnected at the ends.

Chambers.—Transparent plastic is good material because it is impermeable to gases and is worked easily with ordinary woodworking tools. Pieces are cemented together by flowing chloroform along the contacting surfaces with a medicine dropper pipet, or a cement may be made by dissolving scraps of the plastic in chloroform to form a thick paste. This can be applied with a glass syringe, but the glassware must be cleaned with chloroform immediately after use. Surfaces may be freed from grease with carbon tetrachloride.

The three identical chambers for the rat respirometer are made of lu-

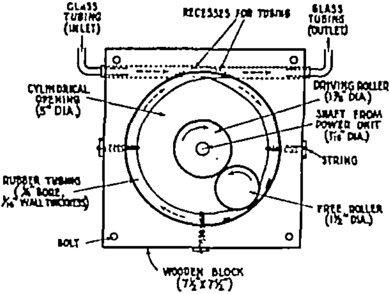

FIG. 2.—Construction of rubber tube pump used to circulate air through the respirometer (Fig. 1). Front cover is removed to show details.

cite tubing of $^1/_8$ in. wall thickness, $4^3/_4$ in. diameter and $6^3/_4$ in. depth. They are closed at the bottom by cementing on a plate of plastic. An overlapping ring of $^3/_8$ in. plastic with a circular opening large enough to admit a rat is sealed to the top.

A rectangular sheet of $^3/_8$ in. plastic of a size to fit conveniently into the water bath is braced with two 1 in. strips of $^6/_8$ in. plastic (Fig. 1). The flat upper surfaces of the top rings of the chambers fit tightly against the underside of this plate and are locked into position readily with a twist. The simple inclined plane locking device is similar to that on pressure cookers. The beveled, sloping edges of the chamber ring fit under three small blocks of plastic cemented to the plate, and when the chamber is turned the surfaces become tightly locked together. The principle of this connection is seen in the circular cover and the lugs on the plastic block in Figure 3.

space that is important in O_2 consumption measurements and that the fluid volume has little influence except as it affects the gas volume.

TABLE 1.—COMPARISON OF GAS AND FLUID VOLUMES AND CORRESPONDING CONSTANTS FOR VARIOUS TYPES OF RESPIROMETERS

TYPE OF RESPIROMETER	VOL. OF GAS SPACE, ML	VOL. OF LIQUID, ML	K	VOL. OF O_2 EQUIVALENT TO 1 MM CHANGE IN MANOMETER FLUID, µl
1. Large (for rats)	3650	60	0.331*	331
2. Small (for mice)	620	30	0.056*	56
3. Large (for aquatic animals, with 1 l of water)	2800	1060	0.257*	257
4. Large (same gas vol. as #3 but no water in animal chamber)	2800	60	0.254*	254
5. Microtype	6	1.6	0.55†	0.55

* For results as ml of O_2.
† For results as µl of O_2.

DETAILS OF USE

Calibration.—To calculate the constant used in computing O_2 consumption it is necessary to know the volume of fluid and of gas space in the system. With the large chambers it is convenient and sufficiently accurate to determine total volume by simply measuring the amount of water required to fill the containers. The volume of the glass and rubber tubing is such a small part of the total that it may be satisfactorily estimated by determining the lengths and multiplying by the unit volumes. The volume of the animal may also be estimated from its weight.

After total volume has been measured it is possible to calculate the constant for the apparatus from the constant volume manometer equation (1):

$$K = \left(\frac{V_G \dfrac{273}{\text{absolute temp. of water bath}} + V_F \alpha}{P_o} \right)$$

where K = constant for the respirometer, V_G = volume of gas space in system in ml, V_F = volume of liquid in system in ml, α = solubility of O_2 in water (ml of gas at N.T.P. dissolved in 1 ml of liquid) (at 15 C, $\alpha = 0.034$; at 20 C, $\alpha = 0.031$; at 28 C, $\alpha = 0.027$; at 38 C, $\alpha = 0.023$), and P_o = 10,000 mm with Brodie's solution as the manometer fluid.‡ If a thermobarometer-corrected manometer reading is multiplied by this constant, K, for the system, the result is the number of milliliters of O_2 consumed for the time interval measured, expressed in terms of normal temperature and pressure.

‡ Brodie's solution is prepared by dissolving 23 g of sodium chloride, 5 g of sodium taurocholate, a few drops of alcoholic thymol solution and some Evans blue or methyl violet dye in 500 ml of water. Filter before use.

microflasks. They are operated with 1 ml of fluid, and total gas space is approximately 6 ml.

The pump is made of plastic and is similar in construction to the larger one (Fig. 2). A plastic roller rolls over five coils of $^{3}/_{16}$ in. thin-walled rubber tubing, one coil for each unit, and forces the air into conical chambers in the transparent block through a hole in each of the covers. Short pieces of glass tubing lead the air from the connections on the covers to the bottoms of conical pyrex cups containing the animal or tissue to be studied. These cups are made from 50 ml centrifuge tubes by cutting off the conical bottom. The air then passes into a side well in the plastic block which contains 0.6 ml of alkali on filter paper and returns

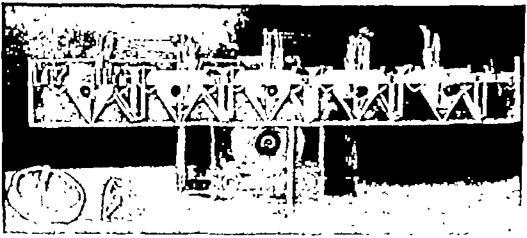

FIG. 3.—Plastic block and pump of the microform of the constant flow respirometer.

through a glass tube to the other end of the rubber tubing at the pump. An opening in the front of each chamber leads to a manometer tube which indicates pressure change. One chamber is run empty to serve as a thermobarometer.

Both block and pump are submerged in a constant temperature water bath, with the pump fitting into a stainless steel box to keep it dry. If humidity is high it is preferable to use brass bushings at the ends of the pump shaft rather than the steel ball bearings illustrated.

The microsystem illustrated is still in an experimental stage of development. It could perhaps be improved and simplified by designing glass flasks that would fit directly onto the manometer tubes and still provide for aeration by flow from the pump. This would allow for more rapid temperature equilibration and eliminate certain connections.

Sensitivity.—The sensitivity of the respirometer varies primarily with the volume of the gas space, and it can be adjusted to the type of animal being studied by altering the size of the chambers. Table 1 indicates the correspondence between gas volume and manometer fluid readings for the three types of systems described. It is obvious *that it is the actual gas*

space that is important in O_2 consumption measurements and that the fluid volume has little influence except as it affects the gas volume.

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5. Microtype	6	1 6	0.55†	0 55

* For results as ml of O_2.
† For results as µl of O_2.

DETAILS OF USE

Calibration.—To calculate the constant used in computing O_2 consumption it is necessary to know the volume of fluid and of gas space in the system. With the large chambers it is convenient and sufficiently accurate to determine total volume by simply measuring the amount of water required to fill the containers. The volume of the glass and rubber tubing is such a small part of the total that it may be satisfactorily estimated by determining the lengths and multiplying by the unit volumes. The volume of the animal may also be estimated from its weight.

After total volume has been measured it is possible to calculate the constant for the apparatus from the constant volume manometer equation (1):

$$K = \left(\frac{V_G \dfrac{273}{\text{absolute temp. of water bath}} + V_F\alpha}{P_s} \right)$$

where K = constant for the respirometer, V_G = volume of gas space in system in ml, V_F = volume of liquid in system in ml, α = solubility of O_2 in water (ml of gas at N.T.P. dissolved in 1 ml of liquid) (at 15 C, α = 0.034; at 20 C, α = 0.031; at 28 C, α = 0.027; at 38 C, α = 0.023), and P_s = 10,000 mm with Brodie's solution as the manometer fluid.‡ If a thermobarometer-corrected manometer reading is multiplied by this constant, K, for the system, the result is the number of milliliters of O_2 consumed for the time interval measured, expressed in terms of normal temperature and pressure.

‡ Brodie's solution is prepared by dissolving 23 g of sodium chloride, 5 g of sodium taurocholate, a few drops of alcoholic thymol solution and some Evans blue or methyl violet dye in 500 ml of water. Filter before use.

Sample calculation: In the large respirometer chambers used for rats, the total volume of both containers, rubber tube pump and connecting glass tubing is approximately 3800 ml. With a 150 g rat in the first chamber and 60 ml of alkaline CO_2-absorbing solution in the tubes in the second, the gas volume is reduced to about 3650 ml. In deriving the constant for a temperature of 28 C, the equation becomes

$$K = \frac{3650\,\frac{273}{301} + (60 \times 0.027)}{10{,}000} = 0.331.$$

The aquatic animal respirometer constant is calculated similarly, with proper allowances being made for the volume of water used.

With the microform of the apparatus (Fig. 3), volume determination with water is not sufficiently accurate and mercury is unsafe because cleaning may not remove it completely from plastic and rubber. However, the differential reading technique in which the volume of fluid in the flask is varied and the response of the manometer column is noted is convenient and accurate to within a few percentage points. (For details of the method, see Umbreit *et al.* (10).)

Reading and calculation.—Since one end of the manometer U-tube attached to the respirometer is open to the air, use of a thermobarometer is required to correct for changes in temperature and barometric pressure. The thermobarometer chamber should contain a small quantity of water to maintain a vapor tension equivalent to that in the measurement system. Each reading of the respirometer is corrected by use of the corresponding thermobarometer reading before it is multiplied by the constant, K, for the system.

Example: In a typical experiment a 155 g rat was placed in the chamber of the respirometer, an oxygen bag connected to the top opening of the manometer tube, and air circulated through the system for 20 min to assure equilibration. The stopcock was then turned 180° to interconnect the respirometer and manometer fluid column, the closed column of fluid was set at the 150 mm mark, and a reading was taken of the open left-hand column. The manometer tube connected to the thermobarometer chamber was also set at the 150 mm mark and its open column was read similarly. After 10 min the closed ends of the two columns of fluid were reset at the 150 mm mark and readings were taken of the left-hand columns. Readings were:

Reading	Respirometer	Thermobarometer
Start	143	151
Ten minutes	22	154
Difference	121	3

Corrected respirometer reading = 121 + 3 = 124.

(Since the thermobarometer fluid moved in the direction opposite that in the respirometer column, the amount of this reading must be added to the difference in the two respirometer readings. When it moves in the same direction it should be subtracted.) Oxygen consumption is then calculated as follows:

(O_2 CONSUMPTION)		(CORRECTED RESPIROMETER READING)		(RESPIROMETER CONSTANT)
x	=	k	$\times$	K
x	=	124	$\times$	0.331
x	=	41 ml O_2 used in 10 min.		

There is some fluctuation in height of the manometer fluid column at the time the roller in the pump passes over the outlets of the rubber tubing. With the large pump the manometer can be read at the middle of the cycle, and with the smaller outfit the pump can be stopped at a neutral position while the reading is taken.

A short lag in response of the manometer fluid to a change in rate of respiration may occur, owing to delay in removal of CO_2. This depends on the rate of circulation of air through the system and is ordinarily unimportant unless there is a sharp change in rate of carbon dioxide output.

Equilibration.—Temperature equilibration with air in the system is rapid, and experiments with mammals may be started 20 min or so after the chambers are immersed in the water bath. With aquatic animals the water should be at about the temperature of the water in the bath when the experiment is started or a long equilibration period may be necessary. Since plastic transmits heat slowly, the block of chambers used in the microapparatus should be allowed to come to water bath temperature before the experimental run is started.

Oxygen equilibrium between water and air is attained rapidly in experiments on aquatic animals. Usually the lag in manometric response to a change in rate of respiration of the animal is insignificant.

Carbon dioxide absorption.—Two tubes of 10 per cent KOH in the center chamber remove CO_2 from the air as it passes through. More absorbing surface is presented if pads of pure cotton gauze (containing no filler or starch) are placed in the upper portion of each tube in such a way that they dip into the alkali. If the rate of respiration is high it is advisable to put a layer of the alkali-soaked gauze on the floor of the second chamber to remove any CO_2 which might pass through the solutions.

When the aquatic animal respirometer is used with sea water, removal of CO_2 results in some increase in alkalinity (11), which may be deleterious if pronounced. A buffer can be added (3), but ordinarily this is unnecessary, since CO_2 production of the respiring material is usually sufficient to maintain the normal pH. When the volume of sea water is relatively large, the change in pH is insignificant for experiments of a few hours' duration, even if there is little CO_2 production.

Replacement of oxygen.—Addition of O_2 to replace that used by the animal is made by means of a rubber anesthesia bag filled with the gas and attached to the top opening of the manometer tube. The system is brought back to original O_2 tension at any time by turning the manometer stopcock and allowing the gas to flow in. If the rubber bag is left on the water under the cover of the constant temperature bath the gas

remains at a temperature so nearly that of the air in the system that a re-equilibration period of about 1/2 min is sufficient after O_2 has been admitted. Some water may be left in the bag with the O_2 to keep the gas saturated, but this is usually unimportant since the amount of gas admitted is slight compared with the total volume of the system.

Respiratory quotient determinations.—The CO_2 output of small mammals can be measured with the constant flow respirometer by absorbing the gas in $Ba(OH)_2$ and titrating with HCl (9). By means of the Y-tube at the inlet of the center chamber (Fig. 1), the air from the container holding the animal may be directed through either the chamber or an auxiliary set of two alkali tubes suspended in the water bath. During the preliminary equilibration period or until the animal becomes quiet the flow of gas is routed through the outer tubes containing 10 per cent KOH solution. At the start of the measurement period the clamp on the tubing is shifted to direct the gas through the center chamber which contains 500 ml of standardized 0.1N $Ba(OH)_2$ solution. At the end of the run the clamp is again shifted, and the $Ba(OH)_2$ is removed and titrated with 0.08N HCl to phenolphthalein indicator. The O_2 uptake is observed for the same period by means of the manometric readings, and from the two determinations the respiratory quotient can be calculated.

The O_2 consumption in milliliters for a given measurement period may be converted to mM of O_2 by dividing by 22.4. To determine mM of CO_2 produced, the difference in the amount of HCl used in neutralizing a "blank" 500 ml sample of 0.1N $Ba(OH)_2$ and that used in neutralizing a sample after a respiration measurement period is noted. Each ml of 0.08N acid thus used is equivalent to 0.04 mM of CO_2. The respiratory quotient may then be obtained by dividing the mM of CO_2 produced by the mM of O_2 consumed.

Example: Observed O_2 consumption of a 155 g rat for 1 hr was 213 ml.

$$213 \div 22.4 = 9.50 \text{ mM of } O_2.$$

To neutralize 500 ml. of 0.1N $Ba(OH)_2$ solution tested as a blank, 623 ml of 0.08N HCl was required. The 500 ml of $Ba(OH)_2$ solution used to absorb the CO_2 produced by the rat required 446 ml of acid for neutralization after the experimental run. The 177 ml difference was due to reaction of the hydroxide with CO_2 from the rat respiration.

$$177 \times 0.04 = 7.08 \text{ mM of } CO_2.$$

The respiratory quotient is then:

$$7.08 \div 9.50 = 0.74.$$

Efficiency of CO_2 removal by $Ba(OH)_2$ depends on the amount of fluid surface exposed to the gas as it passes through the tubes. The rate of gas flow, size of the bubbles and length of the fluid column are the important factors, and these may be varied to insure complete removal. Adequacy of removal may be checked for a particular set-up by ob-

serving whether any carbonate is precipitated in a test $Ba(OH)_2$ solution placed in a trap tube after the collecting solution. Care must be exercised during manipulations to avoid contamination of the $Ba(OH)_2$ solution by CO_2 from the air. Carbon dioxide passes through rubber tubing to some extent, and connections between the animal chamber and the absorbing unit should have as little exposed rubber surface as possible.

Use in cyanide inhibition studies.—The apparatus is particularly well suited for observations of the effect of cyanide on O_2 consumption. Cyanide inhibits tissue oxidations by combining with intracellular heavy metal enzyme catalysts. Sensitivity varies with the type of tissue. In rats, for example, the sensitive nervous system may be inhibited, causing death at a concentration which still permits normal heart action. It may possibly be more accurate to determine the tolerance of the central nervous system to cyanide by observing the physiologic effects of specific concentrations on intact animals, rather than to make inhibition measurements on brain tissue slices.

TABLE 2.—Calcium Cyanide Mixtures for Use at 28 C in Experiments with Mammals

HCN, Ppm	$Ca(CN)_2$ Concentration, M*
600	1.24
500	1.14
400	1.00
300	0.85
200	0.68
150	0.59
100	0.46
75	0.38
50	0.30
25	0.14

* If CO_2-free air is bubbled through a mixture of 10 per cent $Ca(OH)_2$ and the $Ca(CN)_2$ solution indicated in column 2, the outgoing gas will contain the concentration of HCN listed in column 1.

The cyanide gas concentration may be maintained by inserting a bubbler of $Ca(CN)_2$–$Ca(OH)_2$ mixture in the second chamber to add HCN gas to the air after CO_2 has been removed (see pp. 300 ff.). The HCN tension of such a mixture varies with the concentration of $Ca(CN)_2$ and with the temperature (6), and it is thus possible, by use of the proper solution, to maintain a constant concentration of cyanide in the animal chamber and still remove the CO_2 produced. The air entering the second plastic container passes through two tubes of KOH solution, containing cotton pads previously described, blows through alkali-soaked gauze which lines the bottom of the chamber, then bubbles through the $Ca(CN)_2$–$Ca(OH)_2$ mixture. Hydrogen cyanide equilibrium occurs rapidly even though the bubbles are large and stay in the solution only a short time. Table 2 lists the concentrations of $Ca(CN)_2$ that may be used in experiments with mammals.

To check the concentration of cyanide gas in the animal chamber a sample of air may be withdrawn from one of the outlets into a glass

syringe and expelled through a short glass tube into a colorimeter tube containing 3 parts of 0.005M Na_2HPO_4 solution and 1 part of phenolphthalin reagent (4).§ (Withdrawal of air containing cyanide is made directly into the glass syringe from the rubber tubing with no needle between, since there is a rapid reaction when the gas comes in contact with metal.) When 1 part of 0.1 per cent KOH solution is added to the tube the red color which develops is proportional to the concentration of cyanide. The quantity of cyanide in the gas may then be calculated by photoelectric comparison of the sample with the color developed in 0–0.00015M standard solutions of KCN. A cyanide concentration greatly in excess of the 0.00015M capacity of the reacting mixture may result in some inhibition of color development. When the approximate concentration of cyanide in the air is not known, a preliminary test should be made in a tube containing the buffer, reagent and alkali; development of color as the gas is bubbled through is then observable.

A control O_2 consumption determination may be made first with the air passing through only the KOH. Then by shifting a clamp, the flow through the $Ca(CN)_2$–$Ca(OH)_2$ mixture is started, and only a momentary break in the measurement period results.

When aquatic animals are studied a different series of $Ca(CN)_2$–$Ca(OH)_2$ mixtures must be used to maintain HCN equilibrium. Those for experiments in either fresh or sea water at 20 C are listed in Table 3

TABLE 3.—Calcium Cyanide Solutions for Maintaining Cyanide Equilibrium with Water at 20 C

(Approximately 10% calcium hydroxide suspension included in each)

Concentration of HCN in Water, M	$Ca(CN)_2$ Concentration for Equilibrium, M*
0.010	1.41
0.0046	1.23
0.0022	0.87
0.0010	0.56
0.00046	0.31
0.00022	0.16
0.00010	0.080
0.000046	0.048
0.000022	0.026
0.000010	0.014
0.0000046	0.0074

* If CO_2-free air is bubbled through the $Ca(CN)_2$ solutions listed in column 2, it may then be passed through the *HCN solutions listed in column 1 without change of their cyanide concentrations.*

(7). Here again a control determination of O_2 consumption may be made first; then a quantity equal to $^1/_{100}$th the total water volume of a neutralized KCN solution 100 times the desired final concentration is added to the water in the animal chamber through one of the top tubes. At the same time the clamp on the Y-tube (at the outlet of the second chamber) is shifted to make the CO_2-free air bubble through the tube containing

§ The colorimetric reagent is prepared by dissolving 50 mg. of phenolphthalin in 10 ml of ethanol and adding this to 990 ml of 0.01 per cent aqueous $CuSO_4 \cdot 5H_2O$.

the $Ca(CN)_2$–$Ca(OH)_2$ mixture. In this way the gas which returns to the animal chamber is always in cyanide equilibrium with the fluid in it. Even though the experiment is continued for hours, concentration of the experimental fluid will not change.

Solutions which may be used for CO_2 absorption and maintenance of HCN equilibrium in the smaller apparatus (Fig. 3) are given on pages 308 ff. A few drops of these $Ca(CN)_2$–$Ca(OH)_2$ mixtures placed on filter papers in the side wells effectively remove CO_2 and keep the cyanide tension at the desired level. To assure rapid absorption one may use two filter papers, one of which is fluted and fitted into the second one that is rolled.

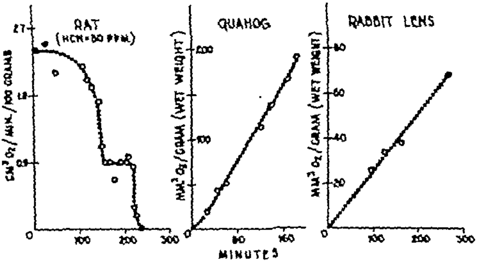

FIG. 4.—Oxygen consumption measurements obtained with the three types of constant flow respirometers described in text.

Illustrative examples: Figure 4 presents results of typical experiments with the various types of respirometers described here. The first curve shows respiration measurements of a 150 g white rat, first for a control period and later when air passing through the chamber contained a concentration of 50 ppm of HCN. Survival of the animal for several hours at a cyanide tension that would cause depression of isolated tissue respiration is possibly associated with detoxication mechanisms in the intact organism.

In the determination represented by the second curve the apparatus contained 1 liter of sea water and a marine shellfish, *Venus mercenaria*. Wet weight of the soft tissues of the organism was 36 g. Oxygen consumption is recorded for a 3 hr period.

The third curve illustrates O_2 uptake by an isolated crystalline lens from the eye of an adult rabbit, in Ringer's phosphate medium in the small apparatus. If the lenses are shaken in an ordinary Warburg manometer for a prolonged period they tend to break up and respiration is no longer normal. The gently flowing stream of bubbles in the constant flow respirometer prevents the capsule from rupturing and still maintains adequate aeration.

It is hoped that the descriptive and experimental detail reported here may show enough of the possibilities of the constant flow respirometer method to suggest its application to a variety of experimental problems.

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RESPIRATION OF TISSUE SLICES

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Introduction.—The tissue slice technique is a method for preparation of groups of cells which are surviving in vitro under controlled conditions and in which the kind, concentration and spatial arrangement of protoplasmic constituents are approximately the same as in vivo (35, 36, 88, 94). Information supplied by metabolic studies on such material is of interest both in its own right and as background for interpretation of data obtained by the more analytical homogenate and isolated enzyme methods. In the latter connection there is much evidence that the normal sequential relations of chemical events in the cell depend in part on the opportunities and limitations imposed by cell structure (8, 22, 46, 82, 87). Accordingly, insight into the manner in which the biochemical potentialities of the cell, revealed by the more analytical techniques, are actually exercised must stem in large measure from studies on intact cells (cf. 22).

Tissue respiration and glycolysis are studied chiefly by means of the manometric methods developed by Warburg and his associates (105) at Berlin-Dahlem and by workers at the Cambridge Biochemical Laboratories (32). There is a large and readily available literature dealing with the manometric method. Basic principles are treated extensively in the reviews of Krebs (73) and Dickens (28) and the monographs of Dixon (32) and Umbreit, Burris and Stauffer (104). An excellent operative discussion is given by Summerson (97). Practical details such as descriptions of manometers, flasks, shaking devices, thermostats and accessory equipment; directions for calibration, use and cleaning of apparatus and preparation of tissue, suspension media, gas mixtures and the like; suggestions for design of experiments and methods for calculation of results are also given by these authors. Several original papers have included critical analyses of various aspects of the technique (15, 23, 80).

This review is not intended to replace the standard references just cited. Rather it is designed to provide useful supplementary information. Methods developed in our laboratory which have been tested and found advantageous, and which have been reported piecemeal or not at all, are described in some detail. In particular the rationale and use of the moist cold box technique for preparation of tissue slices are discussed more fully than hitherto. Factors affecting the choice of suspension medium and of basis of reference for calculation of metabolic data (e.g., dry

It is hoped that the descriptive and experimental detail reported here may show enough of the possibilities of the constant flow respirometer method to suggest its application to a variety of experimental problems.

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The moist cold box is essentially a device for maintenance of low temperature and avoidance of dehydration during the preparation of tissue. Its use is based on the following considerations. The period between the death of the animal and the aeration of the manometer flasks is one of marked tissue anaerobiosis. By means of cold the oxygen demand of the tissues is reduced during this time of inadequate oxygen supply. Furthermore this procedure reduces the rate of all other tissue reactions and thus minimizes the imbalance between the anaerobic and aerobic phases of catabolism which would otherwise occur. There is much evidence favoring the view that low but suprafreezing temperature is more favorable for the survival of ischemic mammalian tissue than the usual body temperature level (3, 4, 13, 14, 33, 47, 96). That the operating temperature of the moist cold box, 5–10 C, does not itself cause tissue damage is indicated by the observation that exposure to lower temperatures for longer times (0.2 C for 60 min) does not impair capacity for oxygen consumption on subsequent rewarming to 37.5 C of slices of rat cerebral cortex (57), kidney cortex (57), liver (59) or heart (60) or of strips of skeletal muscle (69).

A comparison of the respiration of tissue slices which were placed in respirometers as soon as possible after sectioning and after standing for 30 and 60 min in the moist cold box at 5–10 C and in a moist warm box designed after that of Sperry and Brand (97) and kept at 35 C showed the advantage of the former procedure for measurements of tissue respiration (58). Significantly higher rates of oxygen consumption were obtained with rat liver and kidney cortex slices after standing in the cold box than after similar periods in the warm one (58). The differences observed in the case of cerebral cortex slices were in the same direction but were not statistically significant.

The use of low environmental temperature at least during certain stages of tissue slice manipulation is not new (cf. 5). However, as far as we know, the procedure described here, which we have used since 1943, was the first in which all phases of tissue preparation except killing of the animal, excision of the organ and weighing of tissue sections were carried out in a moist cold environment.

TECHNIQUES FOR PREPARING TISSUE SECTIONS

1. *General survey.*—Early workers prepared their material by freehand slicing with a sharp razor (32, 105). The cutting blade was dampened with Ringer's solution. Wetting of the blade in this manner is inadvisable because tissues swell in saline media whether hypo-, iso- or even hypertonic (cf. 37), thus causing erroneous wet weight determinations. Freehand slicing is a fairly satisfactory method for sectioning parenchymatous organs, although not for skeletal muscle (94) or friable tissues such as brain. However, it is rather difficult to prepare sections of uniform thickness in this way. Such uniformity is desirable so that diffusion relationships will remain about the same in successive slices. Accordingly,

weight, nitrogen content and the like) are considered. Finally, there follows this discussion a report by Dr. Robbie on the use of cyanide in studies on tissue respiration in which this important technique is at long last placed on a sound operative basis.

MOIST COLD BOX TECHNIQUE FOR PREPARATION OF TISSUE SLICES

The method of exitus chosen should be quick and should involve a minimum of disturbance of the physiologic state of the tissues. The mechanical problems involved differ somewhat with species. Work in our laboratory has been confined to mice, rats and rabbits. The smaller animals are decapitated with large bone forceps. Rabbits are dispatched by a blow on the back of the neck or by injection of about 30 ml of air into a marginal ear vein. Anesthetics are not used because of their persistent effect on tissue metabolism.

The organs desired are rapidly excised and placed immediately in a small beaker which has been standing well immersed in a tray of finely chopped ice. Beaker and tray are then transferred to the moist cold box (58), where adherent blood is removed by means of filter paper and the tissue is prepared for manometric study as described later. From the time the organ is placed in the box until the beginning of thermoequilibration of the respirometers in a suitable constant temperature bath the tissue is kept in a moist environment at low temperature except for the 10–20 sec required for weighing on a microtorsion balance.

The *moist cold box* is a rectangular chamber made of wood and glass (58). It is large enough to contain all instruments used in preparation of tissues as well as the necessary manometer flasks, media, reagents and the like. The operative portion of the box is approximately 24 in. deep, 30 in. wide, 15 in. high at the back and 10 in. at the front. The sloping top is of glass. The front is designed to admit the hands through holes covered by heavy rubber flaps. The whole box is well illuminated by two fluorescent lamps (20 w) placed behind a frosted glass plate which forms the back of the work chamber of the box. The chamber is cooled by a refrigerator unit placed at the top and back of the operative section. Air in the box is kept moist by distilled water which drips from a separatory funnel over a large gauze wick and is collected in a flat pan. Rate of dripping is controlled by a stopcock on the separatory funnel so that condensation does not occur. Air is circulated through the wick by a small fan which is turned off when tissue is placed in the box. About 1 hr is required to reduce the temperature in the chamber to the operating level of 4–5 C. This is done before the animal is killed. While the box is cooling the glass top and rubber flaps are covered by sheets of insulating material. Necessary equipment and reagents are placed in the box before cooling is begun. For certain procedures the vessels containing solutions are placed in trays of cracked ice. The temperature in the chamber usually rises to about 10 C during the time the tissue is sliced.

tory is the *template technique* of Crismon and Field (25). A battery of such templates, made of rectangular pieces of lucite, 35 × 25 mm and 3 mm thick, is kept on hand. These are ground out in the center so that large ovals are cut in the lower surfaces. These taper off to slots in the upper surfaces. The apertures in the several templates are graded in size so as to be adaptable to a variety of small rounded organs. The entire organ to be sliced is placed on a paraffined stopper (in the moist cold box), the template is gently pressed down on the surface and the tissue which bulges through the slot is shaved off with a safety razor blade (Schick). This blade is not dampened but is wiped off from time to time with cleansing tissue (cf. 37). The practiced worker can prepare very uniform slices in this way. The method is the fastest of any we have tried. It is particularly well adapted to preparation of cerebral cortex slices.

It is fitting to conclude this section by pointing out that it is evident from an examination of the literature as well as from our own experience that the method chosen for preparation of tissue for manometric investigation is largely dictated by the nature of the problem in hand. In general the parenchymatous organs are well adapted for sectioning. Friable tissues like brain offer special problems, as do tissues containing relatively large cells such as cardiac and skeletal muscle. The latter cannot be prepared by slicing if steady states of metabolism are desired.

Slice thickness: Theoretical considerations.—According to Warburg (105, p. 79), the maximal thickness which a tissue slice may attain without limitation of respiration of interior cells through inadequate O_2 supply may be calculated from the formula

$$d' = \sqrt{8C_o \frac{D}{A}},$$

where C_o is the O_2 concentration (in atmospheres) outside the slice, D the diffusion constant in the tissue and A the rate of O_2 consumption in ml/min/ml of tissue. The value of d' is obtained in cm and must be multiplied by 10 to give the equivalent in mm, which is a more convenient measure. When the gas phase in the respirometers consists of O_2 at 1 atmosphere pressure the value of C_o is unity. The value of D may be taken as 1.7×10^{-4} at 38 C (cf. 21, 59, 68). However, this value is based on the figure obtained by Krogh (70) on diaphragm and may not be as representative of the value of D for other tissues as commonly assumed (cf. 11). That $D = 1.7 \times 10^{-4}$ is approximately correct for rat liver is indicated by the good agreement found by Fuhrman and Field (59) between values of d' calculated from the Warburg formula and those obtained by empiric tests.

The basic assumption of the Warburg calculation is that the rate of cell respiration is independent of O_2 pressure until the latter falls to an extremely low level (105, p. 79). In a critical discussion of the validity of this assumption Elliott and Henry (30) pointed out that while at the time it was made the only supporting evidence was provided by work on

various procedures have been devised for facilitating production of sections of suitable and approximately constant thickness. One of the first of these was the method of Deutsch (26), which has been quite satisfactory. The tissue was placed between two microscope slides, the upper one having one frosted surface of medium coarse grain. The lower slide was fixed in a wooden support. A small piece of filter paper, dampened with Ringer's solution (cf., however, 37) was placed on one end of the fixed slide and the piece of tissue placed on it. The frosted slide was then pressed slightly on the tissue, which was cut with a safety razor blade guided by the slide. The frosted surface held the tissue firmly and flat and the process of cutting could be watched because the frosted surface was transparent when wet.

A disadvantage of Deutsch's method is that the uniformity of slice thickness depends largely on the dexterity of the experimenter, although not as much as in freehand slicing. Several instruments have been devised which minimize this difficulty. The slicer designed by Thomas and DeEds (103) was one of the first of these. It consisted of two safety razor blades held apart by a thin metal strip, the whole secured to a handle. The thickness of the slice was determined by that of the metal strip. This instrument is helpful, but its usefulness is limited by the time required to remove the tissue slice in an undamaged condition from between the blades. This difficulty was eliminated in the Terry (102) and Martin (85) *slicers*. Detailed directions for their construction are given in the reports cited. Advantages of the Terry slicer or "safety razor blade microtome" are ease and uniformity of sectioning and ease of removal and economy in use of material. Advantages of the Martin instrument are the unusually long cutting blades, wide range and easy adjustment of the distance between the parallel cutting edges which determines section thickness and ease with which the hinged frame holding the blades can be opened to remove tissue sections and can be returned exactly to the initial cutting position. It is somewhat faster in operation than the Terry slicer. We have used both in the preparation of parenchymatous organs. Of the two we have a slight preference for the Martin slicer unless economy of material is of importance.

The *fresh tissue microtome* described by Stadie and Riggs (99) is somewhat more elaborate than the devices just considered. We cannot present a critical evaluation of this instrument because it has not been available in our laboratory, but its usefulness is shown by the data of Stadie and Riggs (99). When the microtome was designed to cut 0.50 mm sections, the mean thicknesses of a series of liver, kidney, heart and brain slices were 0.51, 0.47, 0.49 and 0.49 mm, respectively, with standard errors less than 5 per cent of these values. Thus the instrument is well adapted for production of very uniform sections. Furthermore it is evidently rapid in operation and economical of material. Careful directions for its construction are given by Stadie and Riggs (99).

A simple procedure which has proved very serviceable in our labora-

2. *Detailed procedures for certain organs.*—The procedures described here are carried out in the moist cold box.

a) *Cerebral cortex.* After decapitation and exsanguination the brain is removed and placed in a chilled beaker and transferred to the moist cold box. The brain is then removed from the beaker and placed on a paraffined stopper. Adherent blood is removed with filter paper and meningeal tissue is stripped off with forceps. Slices are prepared by the template method. Tissue sections are removed from the cutting blade with fine forceps. They are placed in a Petri dish which stands on the surface of a tray of finely cracked ice. The dish is kept humid by a piece of filter paper moistened with Ringer's solution and stuck on the under surface of the lid. The lid is so placed that the dish is partly open to receive the tissue. The slices are arranged in a number of small piles in the Petri dish, corresponding to the number of flasks to be loaded. When a suitable quantity of tissue is on hand the lid of the Petri dish is closed and the ice tray and dish are removed from the cold box. The slices are rapidly weighed by means of a Roller-Smith microtorsion balance. The average load placed in a 15 ml respirometer flask is about 50 mg fresh weight. Aliquot samples are taken from which dry weight is determined (in duplicate or triplicate) by drying to constant weight in an electric oven regulated at 105 C. After receiving the tissue the flasks, which already contain suspension medium, alkali in the central well and the like, are attached to the corresponding manometers and oxygenated at room temperature. When nitrogen-carbon dioxide or oxygen-carbon dioxide mixtures are used the respirometers are gassed in the thermostats set at the temperature chosen for the run. Subsequent procedures are essentially those described by Umbreit, Burris and Stauffer (104).

LIMITING SECTION THICKNESS. The upper limit was calculated from Warburg's formula using the data of Field, Fuhrman and Martin (49) for rat cerebral cortex slices at 37.5 C in an atmosphere of O_2. The maximal value of Q_{O_2} (µl of O_2, measured at standard conditions, per mg initial dry weight per hr) was approximately 12 in 147 adult albino rats of the Slonaker-Wistar strain. The mean wet/dry weight ratio was 5.15. The corresponding value of factor A in Warburg's formula is 3.88×10^{-2}. Using $D = 1.7 \times 10^{-5}$, maximal permissible section thickness for rat cerebral cortex slices in an atmosphere of O_2 is 0.60 mm. Minimal permissible thickness has not been determined. In the light of our experience it must lie at or below 0.25 mm.

b) *Liver.* The excised organ is placed on a linoleum block surfaced with adhesive tape, superficial blood is removed with filter paper, and sections are cut with the Martin slicer in the moist cold box. The sections are trimmed to suitable size with iridectomy scissors and gently blotted with filter paper to remove adherent blood. They are then placed in a cold humid Petri dish as described under "cerebral cortex" for collection and weighing.

LIMITING SECTION THICKNESS. Particular attention should be paid to

micro-organisms, at present there is evidence in its favor obtained by investigations on animal tissues. Thus Warren (109), in "...the first direct and valid study of the effect of very low oxygen tension on an animal tissue" (39), showed that respiration of bone marrow suspensions (measured by the polarimetric method in which the results are not distorted by diffusion and solubility factors) remains constant with decreasing O_2 pressure down to 4 mm Hg or less. Moreover, indirect evidence that liver cells respire at their full rate at very low O_2 tensions is provided by the finding of Fuhrman and Field (59) that the optimal thickness for respiration of liver slices in an atmosphere of O_2 agrees well with the limiting thickness calculated from the Warburg formula (0.55 mm and 0.59 mm, respectively).

Most workers have followed Warburg (105, p. 81) in the assumption that slice thickness is not critical as long as it does not exceed the upper limit, d', for the given tissue and conditions. The general tendency has been to get a slice as thin as possible (94). Shorr (94) has pointed out: "Histological studies, as well as comparative studies of the rate of respiration, show that this is not altogether wise. The superficial layers can be shown to undergo degeneration to variable depth. The thinner the slice the larger is the proportion of damaged tissue. This is particularly important for tissues such as cardiac muscle, where a whole large cell unit at the surface must inevitably undergo degeneration. Thicker slices can be shown to have a higher rate of respiration than very thin ones. The maximum thickness which is permissible is therefore better. Not infrequently thicknesses which exceed the formula behave very well."

In line with Shorr's argument Fuhrman and Field (59) have shown that for rat liver the lower limit of slice thickness is quite critical. Under the conditions of their experiments maximal respiration occurred with a slice thickness of about 0.55 mm, but there was little difference in rate of O_2 consumption between 0.48 and 0.62 mm. Below and above these limits there was a sharp decrease in respiration. Lowered respiration in the thinner slices is presumably due to the larger proportion of damaged cells, and in the thicker ones to inadequate diffusion of oxygen.

Slice thickness: Measurement.—Slices of tissue are trimmed to rectangular form with iridectomy scissors, weighed quickly on a microtorsion balance and spread flat but not stretched on a microscope slide with millimeter cross-section paper underneath. They are examined in transmitted light which has been passed through a cooling cell or is brought into suitable position by means of a curved lucite bar about 1 in. in diameter and 15 in. long. The surface area is determined by counting the number of square millimeters covered by the slice. The volume of the section can be determined with sufficient accuracy from the fresh weight on the assumption that the specific gravity is unity. The mean section thickness is then given by the ratio, volume/surface (cf. 105, p. 81).

94). Steady metabolic states are not obtained with sliced skeletal muscle.

The *muscle strip technique* of Shorr (91, 93, 94) is the best of the methods we have tried. This method was adapted for use with rat skeletal muscle as follows. Immediately after decapitation the hindleg was skinned and amputated at the pelvis in such a way as to include in the preparation the bones of origin of the thigh muscles. The leg was placed in a cold Petri dish resting on ice in a tray and transferred to the moist cold box. Here it was pinned, lateral surface down, to a heavy rubber plate (a dissecting board or linoleum block may be substituted for this). One pin was used to secure the os coccyx, another the knee and a third the distal stump. The soft tissues were then divided with scissors, care being taken that no incision involved the muscles to be used for measurement of respiration. The superficial layers of muscle and fascia over the medial portion of the thigh were stripped off after their attachments were severed near the knee. In the adductor group small strips of fibers were lifted with one tip of a fine forceps (with rounded tips) and their entire length freed from the underlying muscle. Individual groups of fibers 0.3–0.5 mm thick were then separated from each other in a thin band and their ends (connective tissue) severed with iridectomy scissors without grasping with forceps. The strip of tissue was then lifted by the middle as it hung over the forceps and placed in a humid Petri dish resting on a tray of finely cracked ice. The Petri dish and tray were removed from the moist cold box when sufficient material was on hand and the strips of skeletal muscle were quickly weighed (without grasping during transfer) on a microtorsion balance and placed in respirometer flasks containing cold Dickens-Greville Ringer's phosphate solution (29).

LIMITING STRIP THICKNESS. The term "section thickness" is not proper relative to this preparation, which was not sliced at all. The minimal permissible thickness has not been determined; probably it would be that of a single undamaged fiber. The upper limit was calculated from Warburg's formula using unpublished data from our laboratory (69). These data consisted of measurements of respiration at 37.5 C of strips of skeletal muscle prepared as described above from 33 adult albino rats of the Slonaker-Wistar strain. The gas phase in the respirometers was O_2, the liquid phase Dickens-Greville Ringer's phosphate solution (29). Samples from each animal were run in triplicate. Steady states of respiration were obtained for at least the first 40–60 min. Oxygen consumption was calculated from readings obtained during this period of steady state. Mean Q_{O_2} for the entire series (initial dry weight basis) was 3.1, the maximum approximately 5. Mean wet/dry weight ratio was 4.24. From these data (using $Q_{O_2} = 5$), the upper limit of strip thickness calculated from Warburg's formula is 0.83 mm.

f) Other tissues. Tissues other than those just discussed have not been used extensively in our laboratory. A few recent reports on diverse tissues are listed below. The papers cited include some discussion of method. The list is not exhaustive and is intended merely as a useful working

this factor in studies on liver slice respiration because both upper and lower limits are well defined and the permissible thickness range is not wide. For rat liver slices in an atmosphere of O_2 at 37.5 C this range is approximately 0.48–0.62 mm (59). In these circumstances the upper limit calculated from Warburg's formula was 0.59 mm (maximal Q_{O_2} = 7.53, mean wet/dry weight ratio = 3.27, A = 3.83 × 10^{-2}). A Stadie-Riggs microtome built to cut 0.50 mm delivers slices of 0.51 mm thickness (99), which lies in the permissible thickness range.

c) Kidney. After decapsulation this organ is cut in half transversely and sliced with the Terry safety razor blade microtome (102) in the moist cold box. Each slice is gently blotted free of blood with filter paper. Considerable histologic detail can be made out in these sections, so that it is feasible to separate cortical and medullary material with iridectomy scissors (cf. 55). The slices are collected in a cold humid Petri dish as already described.

LIMITING SECTION THICKNESS. The upper limit was calculated from the data of Crismon and Field (25) for rat kidney cortex slices at 37.5 C in an atmosphere of O_2. Maximal Q_{O_2} observed was 20.7; mean wet/dry weight ratio was 4.11. The corresponding value of A is 8.39 × 10^{-2}. Calculated maximal section thickness (d') is 0.40 mm. We have not noted any effect of slice thickness on respiration in kidney sections over the range 0.25–0.40 mm.

d) Heart. The relatively large size of cardiac muscle cells (cf. 93, 94) and the marked sensitivity of cardiac muscle to anoxia (62) are critical factors in preparation of this tissue. Use of comparatively thick slices to minimize the proportion of damaged cells and maintenance of low temperature during preparation are necessary precautions if steady states of respiration are desired.

The excised heart is placed on a paraffined stopper in the moist cold box and sliced by the lucite template method. Sections are gently blotted free of blood with filter paper and collected in a cold humid Petri dish as previously described.

LIMITING SECTION THICKNESS. The upper limit was calculated from Warburg's formula using the data of Fuhrman and Field (60) for rat ventricle slices in an atmosphere of oxygen at 37.5 C. The liquid phase in the flasks was Krebs' Ringer's phosphate (74). Triplicate samples from hearts from 17 adult albino rats of the Slonaker-Wistar strain were run. The mean Q_{O_2} and wet/dry weight ratio for the series were 10.4 and 4.08, respectively. Using 1 and 1.7 × 10^{-5} for C_0 and D, respectively, the calculated upper limit of section thickness is 0.57 mm. Mean rather than maximal Q_{O_2} was used in this calculation because we have some reason to believe that still thicker slices may be desirable.

e) Skeletal muscle. The tissue slice technique is not a satisfactory method for preparing skeletal muscle for metabolic investigation (cf. 35, p. 209). The essential difficulty is that any slicing of skeletal muscle, composed as it is of long fibers, entails extensive cellular damage (93,

94). Steady metabolic states are not obtained with sliced skeletal muscle.

The *muscle strip technique* of Shorr (91, 93, 94) is the best of the methods we have tried. This method was adapted for use with rat skeletal muscle as follows. Immediately after decapitation the hindleg was skinned and amputated at the pelvis in such a way as to include in the preparation the bones of origin of the thigh muscles. The leg was placed in a cold Petri dish resting on ice in a tray and transferred to the moist cold box. Here it was pinned, lateral surface down, to a heavy rubber plate (a dissecting board or linoleum block may be substituted for this). One pin was used to secure the os coccys, another the knee and a third the distal stump. The soft tissues were then divided with scissors, care being taken that no incision involved the muscles to be used for measurement of respiration. The superficial layers of muscle and fascia over the medial portion of the thigh were stripped off after their attachments were severed near the knee. In the adductor group small strips of fibers were lifted with one tip of a fine forceps (with rounded tips) and their entire length freed from the underlying muscle. Individual groups of fibers 0.3–0.5 mm thick were then separated from each other in a thin band and their ends (connective tissue) severed with iridectomy scissors without grasping with forceps. The strip of tissue was then lifted by the middle as it hung over the forceps and placed in a humid Petri dish resting on a tray of finely cracked ice. The Petri dish and tray were removed from the moist cold box when sufficient material was on hand and the strips of skeletal muscle were quickly weighed (without grasping during transfer) on a microtorsion balance and placed in respirometer flasks containing cold Dickens-Greville Ringer's phosphate solution (29).

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f) Other tissues. Tissues other than those just discussed have not been used extensively in our laboratory. A few recent reports on diverse tissues are listed below. The papers cited include some discussion of method. The list is not exhaustive and is intended merely as a useful working

bibliography: bone marrow (106, 108, 112, 114); central nervous system (19, 20, 24); embryonic and fetal tissue (51, 64, 72); eye—cornea (81), lens (50), retina (27, 42); glandular tissue (27, 34, 42); leukocytes (1, 111); lung (34, 95); skin (2, 16, 31); smooth muscle (7, 30); spermatozoa (12, 79, 83); tumors (15, 71 and references in 63).

SUSPENSION MEDIA FOR MAMMALIAN TISSUES

After tissue slices or muscle strips have been cut and weighed they are placed in appropriate manometer flasks (cf. 28, 32, 73, 104, 105) previously filled with the proper volume of a medium suitable for the problem in hand. Choice of medium is usually dictated in part by theoretical and in part by practical considerations. The general purpose is to provide a fluid medium of approximately the same chemical pattern as that of the interstitial fluid, which is essentially an ultrafiltrate of plasma (cf. 61, 65, 86).

1. *Saline media.*—Most measurements of tissue respiration recorded in the literature have been made on tissue in saline media. These media, which may be regarded as modifications of Ringer's solution (cf. 9, p. 211), are designed to resemble the inorganic chemical pattern of mammalian serum. Early workers were content with a pattern that included only the chief cations, sodium, potassium and calcium, with chloride as the anion and small amounts of suitable buffer. However, in view of the importance of extracellular phase magnesium in maintaining intracellular magnesium content (45) and the significance of magnesium as a component of many enzymes (101), this cation is now usually included (29, 74). The mammalian Ringer solutions in current use fall into two categories, depending on whether or not they are intended for use in the presence of CO_2.

a) *Ringer's phosphate solution.* This type of solution is used in measurements of tissue respiration by the direct method of Warburg (32, p. 40) in which CO_2 produced in metabolism is absorbed by alkali so that the tension of this gas in the respirometer is very low (it has a small positive value (98)). Various modifications of Ringer's phosphate solution have been devised (cf. 29, 70, 74, 104). At present there is little basis for choice among these. We have found the media of Krebs (74) and Dickens and Greville (29) very satisfactory in that, with suitable substrate, both support constant rates of respiration (at about the same level) in the tissues we have used for an hour or more. Of the two we prefer the Krebs solution because the potassium concentration (4.8 mE/liter) is closer to that of plasma (approximately 5 mE/kg water (65)) than in the Dickens and Greville medium (2.4 mE/liter). Although this comparison is not quite fair because some of the potassium in plasma is not ionized (65), the level of this cation selected by Krebs seems wiser in view of the significance of potassium in carbohydrate metabolism (41, 53, 65, 66, 78, 89, 114). Furthermore, Krebs' solution has been more widely used, so that data obtained with this medium are more directly comparable with

the bulk of the observations in the literature than if another medium be chosen.

Krebs' Ringer's phosphate solution (74) may be prepared by making up the following stock solutions and adding them together in the proportions indicated under the heading "Parts."

Constituent	Concentration Molar	Concentration %	Parts
1. NaCl	0.154	0.90	100
2. KCl	0.154	1.15	4
3. $CaCl_2$	0.11	1.22	3
4. $MgSO_4 \cdot 7H_2O$	0.154	3.82	1
5. Phosphate buffer*			21

The solution is supersaturated with respect to calcium phosphate and becomes turbid on standing (74). It should be made up just before use (74). Precipitation can be avoided by using half the recommended quantity of calcium chloride solution (115).

A modification of Krebs' Ringer's phosphate solution suggested by Umbreit, Burris and Stauffer (104) consists of solutions 1, 2, 3 and 4, but instead of 21 parts of solution 5 (phosphate buffer), 12 parts are used, together with 1 part of 0.154M KH_2PO_4 (as in the Krebs-Henseleit solution (75)).

b) Ringer's bicarbonate solution. Media containing bicarbonate are used when the metabolism of tissue slices is studied in circumstances in which CO_2 tension in the gas phase of the respirometer is approximately 5 per cent of an atmosphere. This is the case when respiration is measured by the indirect method of Warburg (32), the second method of Dickens and Simer (32) and the like. Bicarbonate media are also used when glycolysis is studied by the manometric technique (cf. 28, 32, 73, 104, 105). We have used the modifications of Krebs and Henseleit (75) and Dickens and Greville (29). We have no empiric basis for choice between these, but prefer the Krebs-Henseleit for the same reasons that led to the choice of Krebs' Ringer's phosphate.

Krebs-Henseleit Ringer's bicarbonate is prepared by using the same stock solutions of NaCl, KCl, $CaCl_2$ and $MgSO_4 \cdot 7H_2O$ in the same proportions as in the Krebs' Ringer's phosphate solution. To these are added 1 part of 0.154M (2.11 per cent) KH_2PO_4 and 21 parts of 0.154M (1.3 per cent) $NaHCO_3$. The bicarbonate solution is gassed with CO_2 for 1 hr before using (75). Stock solution number 5, "phosphate buffer," is not used.

2. *Serum.*—*a) "Neutralized" serum.* This is used when tissue respiration is measured in the presence of alkali to absorb CO_2, as in the direct method of Warburg (32). Under these conditions a gross shift in pH occurs if untreated serum is employed. The essential feature of neutralized serum is that the pH is maintained in the physiologic range in the absence of CO_2 in the gas phase (110). Neutralized serum may be pre-

* The phosphate buffer is prepared by mixing 40 ml of M/4 NaH_2PO_4 and 8 ml of M/1 HCl and diluting this mixture to a volume of 100 ml. The pH is 7.4.

bibliography: bone marrow (100, 108, 112, 114); central nervous system (19, 20, 24); embryonic and fetal tissue (51, 64, 72); eye—cornea (81), lens (60), retina (27, 42); glandular tissue (27, 34, 42); leukocytes (1, 111); lung (34, 95); skin (2, 16, 31); smooth muscle (7, 30); spermatozoa (12, 70, 83); tumors (15, 71 and references in 63).

SUSPENSION MEDIA FOR MAMMALIAN TISSUES

After tissue slices or muscle strips have been cut and weighed they are placed in appropriate manometer flasks (cf. 28, 32, 73, 104, 105) previously filled with the proper volume of a medium suitable for the problem in hand. Choice of medium is usually dictated in part by theoretical and in part by practical considerations. The general purpose is to provide a fluid medium of approximately the same chemical pattern as that of the interstitial fluid, which is essentially an ultrafiltrate of plasma (cf. 61, 65, 80).

1. *Saline media.*—Most measurements of tissue respiration recorded in the literature have been made on tissue in saline media. These media, which may be regarded as modifications of Ringer's solution (cf. 9, p. 211), are designed to resemble the inorganic chemical pattern of mammalian serum. Early workers were content with a pattern that included only the chief cations, sodium, potassium and calcium, with chloride as the anion and small amounts of suitable buffer. However, in view of the importance of extracellular phase magnesium in maintaining intracellular magnesium content (45) and the significance of magnesium as a component of many enzymes (101), this cation is now usually included (29, 74). The mammalian Ringer solutions in current use fall into two categories, depending on whether or not they are intended for use in the presence of CO_2.

a) Ringer's phosphate solution. This type of solution is used in measurements of tissue respiration by the direct method of Warburg (32, p. 49) in which CO_2 produced in metabolism is absorbed by alkali so that the tension of this gas in the respirometer is very low (it has a small positive value (98)). Various modifications of Ringer's phosphate solution have been devised (cf. 29, 70, 74, 104). At present there is little basis for choice among these. We have found the media of Krebs (74) and Dickens and Greville (29) very satisfactory in that, with suitable substrate, both support constant rates of respiration (at about the same level) in the tissues we have used for an hour or more. Of the two we prefer the Krebs solution because the potassium concentration (4.8 mE/liter) is closer to that of plasma (approximately 5 mE/kg water (65)) than in the Dickens and Greville medium (2.4 mE/liter). Although this comparison is not quite fair because some of the potassium in plasma is not ionized (65), the level of this cation selected by Krebs seems wiser in view of the significance of potassium in carbohydrate metabolism (44, 53, 65, 66, 78, 89, 114). Furthermore, Krebs' solution has been more widely used, so that data obtained with this medium are more directly comparable with

nificance. When the heat-stable factor has been identified and made available it may be possible to use a neutralized bicarbonate Ringer's phosphate solution, reinforced with this factor, which is as well suited for in vitro work on tissue metabolism as serum or serum ultrafiltrate and which is easier to prepare.

DETERMINATION OF AMOUNT OF METABOLIZING MATERIAL

1. *Dry weight.*—The results of manometric measurements of tissue metabolism are usually expressed in terms of unit dry weight of tissue (28, 32, 73, 104, 105). There has been some discussion concerning the relative merits of *initial* dry weights, obtained by drying aliquot samples taken at the time sections are cut for respirometry, and *final* dry weights, obtained by determination of the dry weights of residual slices removed from the flasks at the end of an experiment, as bases of reference (15, 42, 43). We are in accord with Burk *et al.* (15) that *initial* dry weight is the better basis. Technical difficulties in recovering tissue from the medium reduce the accuracy of results referred to *final* dry weight. *Final* wet weights are also unreliable both for this reason and because tissue slices swell in isotonic saline media (cf. 37).

In the case of tissues containing relatively large amounts of material that does not contribute to the rate of O_2 consumption a suitable correction should be made. Thus Warren (107) expressed the respiration of bone marrow in terms of fat-free dry weight (calculated from nitrogen determinations), and Fuhrman and Field (59) used glycogen-free *initial* dry weight as a basis of reference for liver slice respiration in the presence of ample exogenous substrate.

DETERMINATION.—Most investigators have determined dry weight by placing tissue samples in a constant temperature oven at 100–110 C and heating until constant weight is obtained (28, 32, 73, 104, 105). Although this method is convenient and sufficiently accurate for most purposes, two errors are introduced when tissue is dried at 100–110 C, "one, a loss of weight due to the volatilization of both fatty and nitrogenous materials; the other, a gain in weight resulting from oxidation. While one may at least partially if not completely offset the other, both can be avoided by drying in a vacuum" (116). The error involved in oven drying at approximately 105 C is not the same for all tissues. In experiments on rat blood, muscle, kidney, liver, brain and skin dried first in the desiccator and then in the oven the changes in calculated water content ranged from 0.4 to 2.1 per cent for the several tissues. "This was probably due to differences in the amount of fat and in the degree of oxidation in the different tissues" (116).

2. *Wet weight.*—Unit wet weight is the proper basis of reference when tissue metabolism in vitro is to be compared with data obtained in vivo by arteriovenous difference estimations or with measurements made on the whole animal (cf. 48). Sections should be cut without moistening the blade to avoid imbibition of water (37, 54–56) and weighed before being

pared by treating the serum with acid and evacuating it to remove the displaced CO_2. The pH is then restored to 7.4 with alkali, and more buffer may be added (52, 84, 107, 110). If complete removal of bicarbonate is desired, the method of Friend and Hastings (52) is recommended. However, partially neutralized serum appears to be preferable for study of tissue respiration (cf. 110, p. 567).

b) Untreated and inactivated serum. Untreated autogenous serum may be used as a suspension medium for tissue slices when the gas phase in the respirometers contains 5 per cent CO_2. When heterologous serum is employed it is previously inactivated by heating for 1–2 hr at 56 C (28, 73). The advantages afforded by use of serum in study of tissue respiration and glycolysis and the difficulties encountered in measurement of aerobic glycolysis in this medium have been discussed by Warren (107, 108, 112) and are considered in the next section.

3. *Balanced salt solutions vs. serum.*—The choice between saline media and serum depends largely on the problem in hand. Saline media offer the advantages of ease of preparation and of known, exact and reproducible composition. The general suitability of the balanced salt solutions of Krebs, Krebs and Henseleit and Dickens and Greville for investigation of tissue respiration is evidenced by: (*a*) Good checks obtained when tissue respiration is measured on successive samples of homogeneous tissue from a given animal. (*b*) Good agreement between measurements of tissue respiration on the same tissue from different animals of the same species and sex and of comparable age and nutritional state. (c) Protracted steady state of tissue respiration observed with sections of certain organs. For example, we have found that the respiration of rat cerebral cortex slices in an atmosphere of oxygen at 37.5 C is constant for more than 4 hr in the Krebs or Dickens-Greville Ringer's phosphate containing 0.2 per cent glucose. Furthermore, the advantages of presence of small amounts of bicarbonate in the medium can be obtained even when the Warburg direct method is used by employing "partially neutralized" bicarbonate Ringer's phosphate solution (110). Last, it appears that such differences in oxygen consumption as may occur when the respiration of tissue slices is measured in the Krebs' Ringer's phosphate and in serum are quantitative rather than qualitative.

On the other hand, serum is the more physiologic medium. It provides the organic as well as the inorganic components of interstitial fluid which is the normal immediate environment of the cell, although there are quantitative differences between serum and interstitial fluid (61, 65, 86). In line with these considerations are reports that the respiration of a variety of tissues is somewhat higher and more constant in serum than in Ringer's solution (cf. 17, 18, 110, 112, 113). Insofar as rabbit bone marrow and guinea-pig liver are concerned these differences are attributable in part to the presence of bicarbonate and in part to heat-stable material present in serum and in serum ultrafiltrate (113). As these two tissues are quite unlike, these two factors may well be of general sig-

Hastings (65, p. 122), is a logical procedure. Use of this standard may clarify relationships which would otherwise be masked when the conventional dry weight basis is employed, because of changes in total solids in the extracellular phase (65). Technical difficulties in determination of the intracellular phase reduce the general usefulness of this standard.

7. *Cell counts.*—The cell count standard is applicable primarily to cell suspensions, for which it is probably the best basis of reference (11). It is difficult to apply this standard to tissue slices in which cell counts are tedious and complicated by the problem of making proper corrections for the cut cells, which are counted twice in serial sections (11).

When a tissue is known to be made up of more than one cell type and the proportion of these can be changed by experimental means it may be possible to estimate the metabolic characteristics of the different types, as Warren (108) has done in studies on rabbit bone marrow. This procedure involves the use of cell counts in combination with dry weight standards.

SYMBOLS EXPRESSING RATES OF TISSUE METABOLISM

Q *notation.*—When dry weight is used as a standard of reference metabolic rates in tissue slices are generally expressed in terms of the Q notation of Warburg (6, 28, 32, 38, 41, 73, 104, 105). The metabolic quotient, Q, was defined as the rate of gas production (+Q values) or consumption (−Q values) in μl, measured at 0 C and 1 atmosphere pressure (N.T.P.), per mg of dry weight per hr. At first the Q notation was used only in relation to reactions in which gas was actually produced or consumed. Later the definition of Q was somewhat extended. The current meaning of Q is given by the expression

$$Q = \frac{\mu\text{l of substance, formed or consumed, at N.T.P.}}{\text{mg dry weight tissue} \times \text{time in hr}}.$$

This term may be applied to any metabolite on the assumption that the substance is in the gaseous state at N.T.P. Various subscripts and superscripts have been used to give explicit meanings. Subscripts refer to the substance formed or consumed, superscripts to the nature of the gas phase in the respirometer. Thus $Q_{CO_2}^{O_2}$ and $Q_{CO_2}^{N_2}$ have been used to denote rates of CO_2 production under aerobic and anaerobic conditions, respectively These terms have sometimes been identified with aerobic and anaerobic lactic acid production because in certain instances lactic acid production appears to account for the CO_2 displacement (using bicarbonate media). However, the manometric measurement of glycolysis is not specific for the acid formed, and lactic acid production does not always account for total acid formation (39–41). Accordingly Elliott and his co-workers (38, 41) suggested that $Q_A^{O_2}$ and $Q_A^{N_2}$ be substituted for $Q_{CO_2}^{O_2}$ and $Q_{CO_2}^{N_2}$, respectively, the subscript "A" merely denoting acid formation without qualitative implications; and that such terms as Q_{LA}, in which "LA" denotes lactic acid production, be used when the

brought in contact with suspension medium (37, 54–56). This gives *initial* wet weight.

3. *Dry weight by protein precipitation.*—The direct use of *initial* dry weight as a basis of reference is possible only if *initial* wet weight determinations are made on the material put in the manometer flasks. When friable tissues are used this may be impracticable. Stadie (98) has developed a procedure suited to these conditions. Protein, present in the medium owing to disintegration of tissue during equilibration in the Warburg vessel, is precipitated by addition of trichloracetic acid to give a final concentration of 7 per cent. The vessel contents are then filtered through a weighed sintered crucible (coarse grade). All visible particles of tissue are transferred to the crucible by washing the vessel two or three times with 7 per cent trichloracetic acid and adding the washings to the crucible. When the crucible has drained thoroughly the tissue on the filter is washed three or four times with 1–2 ml of water to remove salts and trichloracetic acid. The crucible is then dried to constant weight at 110 C. Control experiments have shown that the fluid filtered through the crucible contained no solids other than those present in the original medium and that trichloracetic acid is completely volatilized at 110 C.

4. *Nitrogen determination.*—The nitrogen content of tissue may be used as a standard of reference for metabolic data on the assumption that this content is a reasonable index of the amount of metabolically active material present. This is an alternative to the dry weight standard and, like the latter, is best suited for use with fairly homogeneous tissue (cf. 11). Nitrogen determinations may also be used as a means of estimating fat-free dry weight when the percentage of nitrogen in the fat-free moiety of tissue is known (107).

5. *Nucleic acid standard.*—Berenblum, Chain and Heatley (11) pointed out that dry weight estimations are not a good index of the amount of metabolising tissue when large and variable amounts of metabolically inert material are present, as in skin, breast tissue, necrotic tumors and the like. In these instances the nitrogen standard may also be unsuitable because, like the dry weight standard, it does not differentiate between the cellular respiring elements and the inactive nonrespiring material. It has been suggested that use of the nucleic acid content of the tissue, determined by estimation of nucleic acid phosphorus, as a basis of reference would serve this purpose (10, 11). However, Schneider and Klug (92) have shown that the enzymatic activities of normal tissues are not well correlated with nucleic acid phosphorus. Moreover, nucleic acid phosphorus includes two moieties, representing desoxypentose nucleic acid and pentose nucleic acid, which may vary independently in different tissues. It thus appears that this standard is of doubtful value.

6. *Intracellular phase standard.*—Tissue slice metabolism occurs in the intracellular phase of the tissues studied. Thus expression of tissue metabolism in terms of units of intracellular phase, as suggested by

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substance described by the subscript has been measured by specific chemical methods. In view of the historical connection of the Q notation with manometric measurements it would make for greater clarity if the symbol itself were modified when the metabolic quotient is not based on manometric data. It is suggested that *Q* be used in the latter instance. Thus Q_{O_2}, $Q_A^{O_2}$ and $Q_A^{N_2}$ would denote manometric measurements of respiration, aerobic and anaerobic glycolysis, respectively, while $Q_{LA}^{O_2}$ and $Q_{LA}^{N_2}$ express the rates of aerobic and anaerobic lactic acid formation (in μl as a gas at N.T.P.) per mg of dry weight per hr, determined by chemical methods specific for lactic acid.

Bach (6) pointed out certain limitations in use of the Q notation. If tissue weights and incubation times vary considerably, metabolic activity may not vary directly with tissue dry weight. To make a fair comparison of the data of two tissue slice experiments, one of three conditions must be fulfilled. (*a*) Incubation time must be less than 30 min and wet weight of tissue less than 50 mg, so that small variations in time and tissue weight are linear. (*b*) Relations of time of incubation, tissue weight and the reaction studied must be described from empiric determinations. (*c*) Equal weights of tissue and times of incubation must be used throughout the series of experiments.

U notation.—Q values are measures of rate rather than of total substance consumed or produced. For many purposes an integral unit is more convenient than a differential one. In such cases the term "U," introduced by Staro and Baumann (100), is useful. U may be defined broadly as the number of μl of substance produced or consumed, expressed as a gas at N.T.P., per mg of dry weight tissue in the time *t*. The same rules regarding subscripts, superscripts and the like which govern the usage of Q may be applied to U.

Z notation.—This notation is used when metabolic data are expressed on the cell count basis (77, 90). "Z" is used to denote μl of substance formed or consumed, measured as a gas at N.T.P., per 10^8 cells per hr. The Z notation is thus analogous to the Q notation. Subscript and superscript usage should be the same as in the Q notation.

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substance described by the subscript has been measured by specific chemical methods. In view of the historical connection of the Q notation with manometric measurements it would make for greater clarity if the symbol itself were modified when the metabolic quotient is not based on manometric data. It is suggested that Q be used in the latter instance. Thus Q_{O_2}, $Q_A^{O_2}$ and $Q_A^{N_2}$ would denote manometric measurements of respiration, aerobic and anaerobic glycolysis, respectively, while $Q_{LA}^{O_2}$ and $Q_{LA}^{N_2}$ express the rates of aerobic and anaerobic lactic acid formation (in μl as a gas at N.T.P.) per mg of dry weight per hr, determined by chemical methods specific for lactic acid.

Bach (6) pointed out certain limitations in use of the Q notation. If tissue weights and incubation times vary considerably, metabolic activity may not vary directly with tissue dry weight. To make a fair comparison of the data of two tissue slice experiments, one of three conditions must be fulfilled. (*a*) Incubation time must be less than 30 min and wet weight of tissue less than 50 mg, so that small variations in time and tissue weight are linear. (*b*) Relations of time of incubation, tissue weight and the reaction studied must be described from empiric determinations. (*c*) Equal weights of tissue and times of incubation must be used throughout the series of experiments.

U notation.—Q values are measures of rate rather than of total substance consumed or produced. For many purposes an integral unit is more convenient than a differential one. In such cases the term "U," introduced by Stare and Baumann (100), is useful. U may be defined broadly as the number of μl of substance produced or consumed, expressed as a gas at N.T.P., per mg of dry weight tissue in the time *t*. The same rules regarding subscripts, superscripts and the like which govern the usage of Q may be applied to U.

Z notation.—This notation is used when metabolic data are expressed on the cell count basis (77, 90). "Z" is used to denote μl of substance formed or consumed, measured as a gas at N.T.P., per 10^8 cells per hr. The Z notation is thus analogous to the Q notation. Subscript and superscript usage should be the same as in the Q notation.

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Use of Cyanide in Tissue Respiration Studies

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Inhibition of heavy metal catalysis by hydrocyanic acid is a valuable experimental technique for studying certain problems of fundamental metabolism. The pronounced permeability of the cell membrane to cyanide and the rapid diffusion of HCN through living material make it possible to investigate the respiratory processes of tissues in which the cells are left intact. The compound is active in low concentrations and is relatively specific in action. Its state of dissociation varies insignificantly within the usual biologic pH range.

Although these characteristics make cyanide a useful analytic tool for cellular respiration studies, lack of adequate control of concentration has made much of the previous research quantitatively incorrect. This difficulty arises chiefly from the volatility and acid nature of HCN gas. Unless proper techniques are used most of the HCN is driven off while the manometer flask is being gassed. If a simple alkaline solution is used in the center well to absorb the CO_2 produced by respiration, the HCN is rapidly absorbed also. Recent developments have made it possible both to measure and to control the concentration of cyanide in manometric experiments (1, 2), and some of the more essential points of these data are presented here.

Necessity for controlling cyanide concentration.—Inhibition of certain enzymatic processes by cyanide in low concentrations is due to the combination of cyanide with heavy metal catalysts. If this combination is irreversible or has a low rate of dissociation a quantitative study re-

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easier to prepare mixtures with known tensions; it may be used to maintain equilibrium with HCN solutions as high as 10^{-2}M; it provides a steady source of HCN even though gas is given off or CO_2 absorbed. The constant tension is a result of uniform pH, which is in turn dependent on the constant concentration of the dissolved $Ca(OH)_2$. Since the hydroxide is only slightly soluble and a large excess is kept in suspension on the center well filter paper, a reserve is readily available to replace that lost by precipitation of $CaCO_3$ on CO_2 absorption. Conversely, since the solution is saturated, any hydroxide formed by hydrolysis of

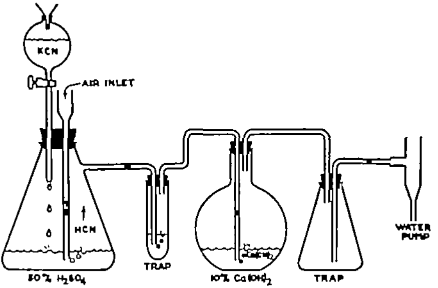


FIG. 1.—Apparatus used in synthesizing $Ca(CN)_2$. (See details in text.)

$Ca(CN)_2$ when HCN is given off precipitates, and again a constant pH is maintained.

Table 1 lists the experimentally determined concentrations for KCN–KOH and for $Ca(CN)_2$–$Ca(OH)_2$ center well mixtures that may be used to absorb CO_2 and to maintain proper HCN equilibrium.

PREPARATION OF CALCIUM CYANIDE

A concentrated solution of $Ca(CN)_2$ in $Ca(OH)_2$ may be prepared simply and with reasonable safety by means of the set-up illustrated in Figure 1 (5). Hydrogen cyanide is generated by dropping KCN solution into 50 per cent H_2SO_4 and the gas is led through a water trap into a $Ca(OH)_2$ suspension. Since the system is attached to a water pump and kept partially evacuated, none of the poisonous gas escapes into the room, but it is well to keep the generator in a well ventilated place. When the air flows through the system as illustrated the long inlet column acts as a safety valve. If for any reason the suction from the water pump fails, the pressure due to the generating HCN will cause the acid to back up in

quires only that the initial concentration of HCN be correct. However, the lability of the heavy metal cyanide compound, as indicated by rate of recovery of respiration after removal of the free HCN, varies considerably in different tissues. The O_2 consumption of the sand dollar egg remains depressed after a short exposure to 10^{-4}M HCN (3); the rabbit cornea recovers completely soon after treatment with 50 times this concentration (7). Yet in both tissues the O_2 consumption is almost entirely inhibited by cyanide and it is probable that a heavy metal is involved in the cellular oxidation process. Therefore to study adequately the comparative action of cyanide the HCN gas tension in the manometer flask must be kept constant throughout the experimental measurement period.

Equilibrium with center well solutions.—The volatility and acid nature of HCN cause rapid transfer of the gas from the experimental fluid to the alkaline center well solution (8, 2), and unless there is a counterbalancing HCN tension, the concentration of cyanide in the fluid may drop rapidly.

Two types of center well mixtures that have been used successfully to absorb the CO_2 produced in respiration and still maintain proper HCN tension are KCN–KOH and $Ca(CN)_2$–$Ca(OH)_2$. Although the former solution is somewhat simpler to prepare, its use is restricted to the lower ranges of HCN concentration, and the amount of CO_2 which may be absorbed without change in HCN tension is limited. Also, a variation in the amount of alkali in different lots of stock KCN crystals causes a variability in the pH of the KCN–KOH preparation that is difficult to determine precisely.

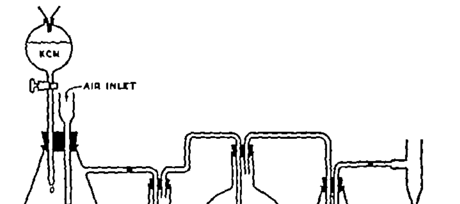
The $Ca(CN)_2$–$Ca(OH)_2$ system is preferable in several ways: it is

TABLE 1.—Composition of Center Well Fluids for Manometric Experiments with Cyanide*

Exper. Fluid Concentration of HCN (M)	Center Well Solution					
	Calcium Cyanide (M)			Potassium Cyanide (M)		
	20 C	25 C	37.5 C	20 C	25 C	37.5 C
1×10^{-2}	1.58	1.62	1.45	...	...	...
0.46×10^{-2}	1.20	1.26	1.07	...	...	...
0.22×10^{-2}	0.71	0.78	0.63	...	...	...
1×10^{-3}	0.38	0.42	0.32	...	...	...
0.46×10^{-3}	0.19	0.21	0.16	5.5	4.8	4.3
0.22×10^{-3}	0.091	0.10	0.078	2.5	2.1	1.9
1×10^{-4}	0.046	0.051	0.038	1.1	0.93	0.83
0.46×10^{-4}	0.022	0.025	0.019	0.50	0.43	0.36
0.22×10^{-4}	0.011	0.013	0.0091	0.22	0.19	0.17
1×10^{-5}	0.0054	0.0063	0.0046	0.10	0.085	0.078
0.46×10^{-5}	0.0026	0.0031	0.0022	0.047	0.039	0.033
0.22×10^{-5}	0.0012	0.0014	0.0011	...	...	...
1×10^{-6}	0.0006	0.0007	0.0005	...	...	...

* $Ca(CN)_2$ solutions contain 10 per cent $Ca(OH)_2$ suspension; KCN solutions contain 0.5M KOH. KCN–KOH solutions for HCN concentrations of 10^{-3} or higher are unsatisfactory because of the rapid change which occurs when CO_2 is absorbed.

easier to prepare mixtures with known tensions; it may be used to maintain equilibrium with HCN solutions as high as 10^{-3}M; it provides a steady source of HCN even though gas is given off or CO_2 absorbed. The constant tension is a result of uniform pH, which is in turn dependent on the constant concentration of the dissolved $Ca(OH)_2$. Since the hydroxide is only slightly soluble and a large excess is kept in suspension on the center well filter paper, a reserve is readily available to replace that lost by precipitation of $CaCO_3$ on CO_2 absorption. Conversely, since the solution is saturated, any hydroxide formed by hydrolysis of



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$Ca(CN)_2$ when HCN is given off precipitates, and again a constant pH is maintained.

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the column and block off escape to the air. When there is sufficient pressure in the flask the drops of KCN solution coming through the narrow tubing will be held back also and the generation stops.

The water trap prevents contamination of the hydroxide by fumes from the strong acid. The flask containing the $Ca(OH)_2$ should be kept in a container of cold water while the gas is passing through, since a warm, concentrated solution of $Ca(CN)_2$ tends to form polymers. An excess of $Ca(OH)_2$ must always be present also to retard polymerization.

Example: In a typical preparation of $Ca(CN)_2$, 25 g of 95 per cent KCN in 75 ml of water was added dropwise to 100 ml of 50 per cent H_2SO_4 over a 30 min period. Aeration was continued for an additional 30 min. The gas was collected in 100 ml of 20 per cent $Ca(OH)_2$ suspension. At the end of the period the yield was 110 ml of 1.0M $Ca(CN)_2$.

A 0.2 ml blood sampling pipet with a rubber bulb attached is a convenient means of safely removing a sample of the strong cyanide solution for analysis. The sample is diluted with glass-distilled water (copper-free) and the concentration measured by means of the phenolphthalin test (1). For the phenolphthalin reagent for colorimetric determination of cyanide concentration see page 280 (footnote). Two parts of the diluted cyanide sample is then added to a colorimeter tube containing 1 part of reagent and 1 part of 0.015M Na_2HPO_4. One part of 0.1 per cent KOH solution is added, the red color is measured in a photoelectric colorimeter after 1 min, and the concentration is determined by comparison with a curve obtained from known concentrations of KCN solutions. Since 10^{-4}M is about the maximal concentration that may be measured conveniently, the $Ca(CN)_2$ sample should be diluted about 50,000 times.

The $Ca(CN)_2$–$Ca(OH)_2$ center well mixtures may be prepared from

TABLE 2.—DATA FOR PREPARATION OF CENTER WELL MIXTURES FOR USE IN CYANIDE EXPERIMENTS AT 37.5 C*

Calcium Cyanide Concentration		Mixture	
Final (M)	Stock (M)	$Ca(CN)_2$ (ml)	10% $Ca(OH)_2$ (ml)
1.45	1.45	12.0	0
1.07	"	8.9	3.2
0.63	"	5.2	6.8
0.32	"	2.7	9.4
0.16	"	1.3	10.7
0.078	"	0.65	11.4
0.038	0.078	5.8	6.2
0.019	"	2.9	9.1
0.0091	"	1.4	10.6
0.0046	"	0.71	11.3
0.0022	0.0046	5.7	6.3
0.0011	"	2.0	9.1
0.0005	"	1.3	10.7

* Column 1 gives concentration of $Ca(CN)_2$ in the mixture (from Table 1). Column 2 lists stock concentration of $Ca(CN)_2$ to be used. If this stock solution is mixed with $Ca(OH)_2$ in proportions shown in columns 3 and 4, the final concentration is that listed in column 1.

the stock solution by mixing the proper amounts of $Ca(CN)_2$ with $Ca(OH)_2$ suspension in 15 ml centrifuge tubes. The proportions for 37.5 C are indicated in Table 2, and for other conditions similar tables may be devised from the data given in Table 1. Mixtures in tubes stoppered with rubber stoppers and kept refrigerated at slightly above 0 C will keep for a month or more. Decomposition results in the formation of ammonia, and if the gas above a solution rapidly turns damp litmus paper blue a new preparation should be made.

Neutralization of cyanide.—Hydrolysis of potassium or sodium cyanide makes a concentrated solution of either of these salts highly alkaline. To add an unneutralized solution of more than 10^{-4}M concentration to

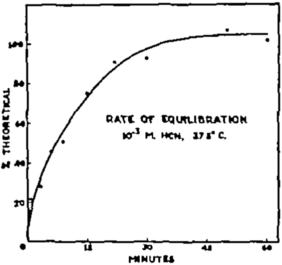

FIG. 2.—Measurement of rate of saturation of the experimental fluid in a Warburg manometer flask containing $Ca(CN)_2$–$Ca(OH)_2$ center well mixture.

a tissue may result in a considerable shift in pH, and the effects obtained may in some measure be due to the alkalinity rather than to the specific action of the cyanide. The shift in pH will depend on the buffering capacity of the physiologic fluid as well as on the concentration of the alkaline cyanide, and the extent of the error involved depends on both of these factors. The simplest procedure is to neutralize a 10^{-1}M solution with HCl, observing the end-point by removing a drop occasionally and testing it with phenol red indicator. This stock solution is then diluted as needed with buffered saline and added to the manometer flasks immediately before the start of the experiments. If it is refrigerated and well stoppered it will keep for weeks with no significant change in concentration.

Rate of equilibrium attainment.—The amount of cyanide in the gas in the manometer flask depends on the HCN tension of both the experimental fluid and the center well solution. At equilibrium the two are, of course, equal. The rate of attainment of this equilibrium level depends on

the temperature and on the volume and exposed surfaces of the two fluids. At 25 C it takes approximately 1 hr for HCN gas from 0.6 ml of center well solution to saturate 3 ml of experimental fluid in a 17 ml Warburg manometer flask. At 37.5 C, however, an approximate saturation is reached in 20 min (Fig. 2). This is of considerable importance and *convenience in experiments at this temperature since it permits one to* set up the manometer flask with cyanide in the center well only. By the time a 20 min equilibration period has elapsed the cyanide concentration in the experimental fluid is close to the proper level. This saves the time which would be occupied in preparing the proper dilute HCN solutions and assures that the experimental flask fluid is different from the control only in the presence of HCN.

Passage of HCN into an alkaline center well solution when the

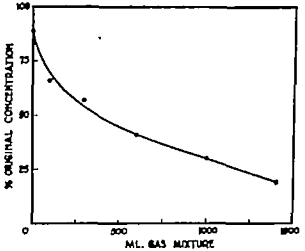

FIG. 3.—Results of experiment demonstrating escape of HCN from flasks of a Summerson differential manometer as 95 per cent O_2–5 per cent CO_2 gas mixture is passed through.

experimental fluid contains cyanide is equally rapid at 37.5 C. It is possible to measure recovery by simply replacing the center well $Ca(CN)_2$–$Ca(OH)_2$ with $Ca(OH)_2$. By the time temperature equilibrium is reestablished the HCN level in the fluid is about as low as it would have been if the tissue had been washed.

Loss of HCN on aeration.—The concentration of cyanide in a $Ca(CN)_2$–$Ca(OH)_2$ center well mixture is more than 200,000 times that in an equivalent volume of air in equilibrium with it at 25 C. This provides a tremendous reserve of HCN which may escape *before a change in the* ultimate concentration is apparent. If flasks are gassed with oxygen at the start of the experiment there is seldom more than a maximum of 1500 ml of gas passed through any one flask. If all gas which passes through comes into HCN equilibrium with the center well solution, and if this amount of cyanide is removed from the system as the gas is blown out,

the change in concentration of the center well is still only about 1 per cent. This is insignificant, and loss of HCN due to gassing can be ignored in a system containing a balanced center well.

In the Warburg indirect method, which does not depend on alkali absorption of CO_2, or in the Summerson differential technique in which CO_2 is not absorbed until the end of the experiment, the error caused by blowing off of HCN on aeration may be of great importance. Figure 3 illustrates determinations of concentration of the fluid in Summerson manometer flasks through which a stream of 95 per cent O_2–5 per cent CO_2 was passed at the rate of 1 liter in 5 min. The flasks originally con-

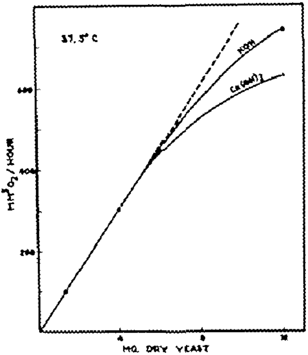

FIG. 4.—Carbon dioxide absorbing capacity of $Ca(OH)_2$ solution compared with 10 per cent KOH at 37.5 C; 0.6 ml. of absorbing fluid on two filter papers in center well; yeast in 3 ml of M/50 KH_2PO_4 buffer in larger chamber of flask.

tained 1 ml of 10^{-3}M HCN solution, and the curve shows the decrease in concentration with increasing volume of gas passed through. Aeration with 1400 ml of the gas mixture reduced the cyanide concentration to only 20 per cent of the original value. To prevent this drop, the incoming gas may be passed through two vessels containing several hundred ml of HCN solution of the desired concentration and kept at the same temperature as the water bath. The importance of this precaution is illustrated by results obtained with rat retinas. Although it had previously been reported that the rat retina was not inhibited by HCN in a bicarbonate medium, recent work in which the cyanide concentration was kept constant by saturating the incoming O_2–CO_2 mixture as described

above showed that the degree of inhibition in bicarbonate was about the same as that in phosphate buffer (6).

Carbon dioxide absorption.—To be satisfactory for manometric use the center well fluid must remove the CO_2 produced during respiration rapidly enough to make the manometric reading a true measurement of O_2 consumption. Since the solubility of $Ca(OH)_2$ is low, its ability to remove CO_2 from the air is limited compared with KOH. At 28 C, 0.6 ml of 10 per cent $Ca(OH)_2$ suspension on filter paper in the manometer center well is able to remove quantitatively about 100 mm^3 of CO_2 per hr. This is equivalent to about half the rate of respiration ordinarily regarded as the maximum in manometric work, and the amount of respiring material should be adjusted with this limitation in mind.

At 37.5 C, however, the rate of CO_2 absorption by the center well $Ca(OH)_2$ is considerably increased. Figure 4 shows results of an experiment in which O_2 uptake of varying quantities of yeast cells was observed with either 10 per cent $Ca(OH)_2$ or 10 per cent KOH in the center well. The respiration with the $Ca(OH)_2$ suspension follows a straight line to a value higher than 300 mm^3 of O_2/hr, indicating that at this temperature CO_2 removal is completely satisfactory.

To help in keeping the maximal amount of center well fluid exposed to the gas in the manometer flask it is well to use two filter paper squares, one rolled into a cylinder and the other fluted by folding and placed inside the cylinder. This aids in more rapid CO_2 absorption and attainment of HCN equilibrium.

Analysis of fluid.—For cyanide solutions of 10^{-4}M or higher, it is possible to withdraw a sample of fluid from the flask and analyze it by the phenolphthalin method described earlier. In this way the correctness of the HCN equilibrium between the center well solution and the experimental fluid can be determined directly. Some cells are inhibited by HCN in concentrations of less than 10^{-4}M, and a biologic method (3) has been used in checking the accuracy of the mixtures below this point in Table 1.

The phenolphthalin technique is not always applicable to solutions which contain tissues, since certain oxidizable substances may interfere. For measurements of this sort it is possible to use a combined aeration-vacuum distillation method in which the cyanide is removed while the fluid is warm and under low pressure and is collected in a solution of the phenolphthalin reagent (4).

The amount of cyanide recoverable from a given volume of fluid in a manometer flask containing tissue may not be that which would theoretically be expected from the HCN tension of the fluid; some of the cyanide may be tied up or adsorbed by the tissue and be released in the analysis process. A properly balanced center well solution provides only for maintenance of uniform HCN tension. The actual cyanide concentration in the cell probably depends on a number of other factors.

Use with tissues.—An example of the use of the $Ca(CN)_2$–$Ca(OH)_2$

center well mixtures in mammalian tissue experiments is given by the curve in Figure 5. Rat spleen slices were prepared with the usual precautions regarding optimal tissue thickness, rate of shaking, gassing with oxygen, etc. Cyanide was added by means of the center well solution only, and measurements were continued for 1 hr after the preliminary equilibration period. It is apparent that although 10^{-6}M HCN has no effect, 10^{-3}M HCN inhibits O_2 consumption almost completely.

In comparing the effect of cyanide on different tissues, several aspects of its action may be investigated. Behavior of the cells with various substrates and without added substrate may be observed. The depression produced by constant concentrations of HCN may be compared with the recovery which may occur on removal of the cyanide. A wide range of

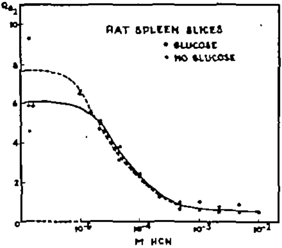

FIG. 5.—Oxygen consumption of rat spleen slices in various concentrations of HCN. $Ca(CN)_2$–$Ca(OH)_2$ mixtures in center wells to maintain HCN equilibrium and absorb CO_2.

concentrations should be employed to determine the relative sensitivity of the particular tissue examined. Although a complete interpretation of certain of these observations is not apparent at present, the use of quantitative techniques may prove to be unexpectedly valuable.

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above showed that the degree of inhibition in bicarbonate was about the same as that in phosphate buffer (6).

Carbon dioxide absorption.—To be satisfactory for manometric use the center well fluid must remove the CO_2 produced during respiration rapidly enough to make the manometric reading a true measurement of O_2 consumption. Since the solubility of $Ca(OH)_2$ is low, its ability to remove CO_2 from the air is limited compared with KOH. At 28 C, 0.6 ml of 10 per cent $Ca(OH)_2$ suspension on filter paper in the manometer center well is able to remove quantitatively about 100 mm^3 of CO_2 per hr. This is equivalent to about half the rate of respiration ordinarily regarded as the maximum in manometric work, and the amount of respiring material should be adjusted with this limitation in mind.

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Use with tissues.—An example of the use of the $Ca(CN)_2$–$Ca(OH)_2$

THE HOMOGENATE TECHNIQUE

VAN R. POTTER

PRINCIPLES

1. *Terminology.*—The term "homogenate" was introduced in 1941 by Potter (22) to refer specifically to suspensions of animal tissues that had been ground in the all-glass "homogenizer" described by Potter and Elvehjem in 1936 (28). The term homogenate has since been used by various investigators to refer to tissue preparations that have been ground in a mortar, with or without sand, or disintegrated in a Waring blendor or produced by methods not described. Although such preparations are probably no less appropriately called homogenates than those made in the all-glass homogenizer, it seems desirable to promote a nomenclature that is as meaningful as possible, and it is suggested that the method of preparation be specified. We recommend use of the original device because of the all-glass construction and consequent freedom from metal or other contamination. All the methods described here apply only to homogenates prepared in glass homogenizers.

The chief significance of the term homogenate is that it serves to distinguish the preparation from slices, minces and extracts. In the slice technique, *an attempt is made to obtain surviving groups of cells* without loss of in vivo properties, whereas the homogenate technique accepts the fact that the cells in the tissue are no longer living and attempts to obtain surviving groups of *enzymes* without loss of in vivo properties. Minces have been employed in lieu of slices or in the case of tissues where slicing seems at best a poor compromise, and there seems to be no good reason for their continued use, although much of our present knowledge was derived from studies with minces and they may still have a place for studies on muscle. The homogenate technique differs from enzyme studies formerly made with "extracts" because it retains all of the tissue and is intended to yield *quantitative* data as to the concentration of enzymes in the tissue. Studies made with extracts are sometimes useful in revealing the presence of enzyme activity but are not suitable for comparative studies, because the relation between the amount of enzyme in the extract and the amount in the original tissue is not measured and it cannot be assumed that this relation will remain the same from one instance to another. However, work with extracts is sometimes necessary to obtain a demonstration of enzyme activity, particularly if uncontrollable side

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is inactivated at pH 6.4, with the result that glycolysis becomes impossible, whereas Green was dealing with oxidative reactions in the Krebs cycle.

Comment by H. G. Wood

The inactivation occurred during the gassing with 5 per cent CO_2–95 per cent N_2 prior to mixing of the homogenate with the substrate and buffer. The preparation was active if bicarbonate was added to the homogenate at the time of gassing.

In the homogenate technique, the autolytic reactions during grinding are decreased to some extent by two means, *cold* and *dilution*. The tissues are removed from the animal as quickly as possible and a sample weighing 1 g or less is placed in a beaker of isotonic medium kept at 0 C with cracked ice. The chilled tissue is quickly weighed and dropped into a tube containing a known amount of cold homogenization medium, and more medium is added to make a total of 9 volumes of medium per weight of tissue. The tissue is then homogenized in the cold in a tight-fitting homogenizer. The rapid dispersal of the tissue to a tenfold dilution decreases the rate of glycolysis and also dilutes the slight amount of acid that is formed so that, in our experience, the pH never drops below 7.0. For most purposes the resulting homogenate is stable at 0 C for as much as 24 hr, but in certain instances it should be used as quickly as possible. Stability is greatly influenced by the homogenization medium (see below).

The dilution of the homogenate itself (1:10) is increased another tenfold when the homogenate is added to the reaction mixture since the usual amount of homogenate per Warburg flask is 0.3 ml in 3.0 ml total volume. Thus the tissue is at a dilution of 1:100, and the various metabolites are $^1/_{100}$th as concentrated as in the original cells. Since in the cells the substrates are in general the chief limiting factors in the reaction rate (see Conclusion, p. 335) this dilution has the effect of lowering many of the reactions to 1 per cent of their in vivo rate, and in systems with several components the effect of dilution is still greater, so that many phases of metabolism are practically at a standstill.

Thus use of cold and of rapid dilution has the effect not only of preserving the enzymatic components of the cells but of stopping the complex of metabolic reactions by lowering the concentration of substrates and cofactors.

The next step in use of the homogenate is the selective activation of the enzyme system under study. With all of the enzymes lacking substrates, the addition of one substrate will at first affect only those enzymes which act on that particular compound. Furthermore, most of the systems with which we deal have several components, and the addition of the special cofactors for a given system will tend to make one particular reaction the rate-limiting one. Thus by *selective activation* one can reconstruct the particular phase of metabolism that one wishes to study

reactions can be eliminated by fractionating the tissue. In this instance, the measurement of activity in the whole homogenate is recommended, since it shows whether anything has been accomplished by the fractionation. Thus the homogenate forms an excellent starting material for work with extracts, and in the discussion to follow, the word homogenate will be taken to mean *whole* homogenate, which means that it contains *all* of the tissue sample. Any derived fractions will be called "extracts" or "residues," with the understanding that when an extract is obtained from a homogenate, a residue remains.

When a given enzyme system is under study, the substance on which it acts is added to the mixture and is called the "primary substrate." It is converted to a substance which is the "product" of the primary enzyme. *If this "product" is acted on by additional enzymes, the "product"* will be called a "secondary substrate" and the additional enzymes will be referred to as the "secondary enzyme system."

Most of the enzyme systems have several components, referred to as coenzymes, activators, carriers and supplementary enzymes, as the case may be.

2. *Nature of the homogenate.*—When a sample of whole tissue is homogenized, the cells are not converted into a solution of enzymes. The majority of the cells in a tissue are disrupted, but the nuclei appear to remain. Furthermore, the particulate components of the cytoplasm remain as discrete particles. The homogenate is therefore a suspension of nuclei and cytoplasmic particles in a diluted version of the soluble components of the cell. It must also be recognized that it is a mixture of *tissue* elements as well, and that the contents of the cells of various types are indiscriminately mixed, along with varying amounts of connective tissue, blood vessels and unclassified cellular and tissue debris that may or may not be metabolically active. At first thought one would expect such a mixture to be practically useless for the study of intracellular processes; and it is true that the preparation of homogenized tissue per se is actually of little value for biochemical studies. The usefulness of the homogenate technique depends on the success with which certain principles are kept in mind. It is evident that knowledge of the nature of the tissue sample must be applied in the interpretation of the results just as in the case of the slice technique, but the other principles of the homogenate technique need to be stressed in more detail.

3. *Principles of dilution and selective activation.*—It is important to bear in mind that as soon as a tissue is disorganized by homogenization, autolytic reactions of all types set in rapidly, so that any study dependent on the maintenance of in vivo conditions must be carried out so as to minimize the effect of the autolytic reactions. These reactions include production of lactic acid and destruction of various phosphorylated activators or cofactors. Work by Utter (40) and by Green (5) has indicated that certain enzyme systems might be inactivated by acid produced when the tissue is ground. Utter reported that phosphofructokinase

is inactivated at pH 6.4, with the result that glycolysis becomes impossible, whereas Green was dealing with oxidative reactions in the Krebs cycle.

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and, in effect, isolate the reaction while leaving the enzyme in a complex milieu.

4. *Control of side reactions.*—With conversion of the primary or added substrate to its product, a second enzyme system is given a substrate on which to act. But by omitting the cofactors of the second system or by inactivating the factors already present one can frequently limit the reaction to the primary conversion. Thus in the succinic dehydrogenase system (35) succinate is converted to fumarate, which is hydrated by fumarase to yield malate. The problem is to prevent malate oxidation, mainly because its oxidation product (oxalacetate) inhibits succinate oxidation. Omission of the coenzyme for malic dehydrogenase helps to prevent malic oxidation, but in addition it is necessary to add calcium ions to increase destruction of the coenzyme and obtain maximal activity of the succinoxidase system.

In the malic system the situation is quite the opposite (20). The coenzyme is added and calcium ions are omitted. Also, nicotinamide is added to decrease destruction of the coenzyme by nucleotidase. In the malic system the secondary substrate is oxalacetic acid. Oxidative removal of this compound is minimized by omitting magnesium and adenosine triphosphate from the reaction mixture, but since the accumulation of oxalacetate inhibits malate oxidation it is removed by a transamination with glutamic acid.

Each system presents a special problem, but the principles are the same: the primary enzyme system is activated by the addition of the appropriate cofactors, and the secondary enzyme systems are inactivated if possible either by omission of cofactors or by any means available.

5. *Choice of media for homogenization.*—The problem of disrupting cells without damage to the cellular contents has not been completely solved, but considerable progress has been made, and at present we employ just two alternative media, either distilled water or alkaline isotonic KCl. Since we are dealing with intracellular elements it is no longer possible to pattern the composition of the medium on extracellular fluids. The use of KCl instead of NaCl is largely dictated by the fact that potassium appears to predominate over sodium inside the cell. In addition, we have obtained better results with potassium in certain reaction mixtures than when it was omitted. Racker and Krimsky (31) reported that potassium is definitely superior to sodium in their glycolytic system.

The general rule seems to be that water is the best homogenization medium when maximal disruption is desired and when it is not accompanied by inactivation of enzyme systems, whereas alkaline isotonic KCl is preferable when certain cellular elements must be maintained intact. Utter, Reiner and Wood (41) obtained maximal activity with water homogenates when studying glycolysis, and we have obtained maximal activity in the malic dehydrogenase and succinic dehydrogenase systems using water homogenates. On the other hand, the oxalacetic oxidase sys-

tem is largely inactivated by homogenization in water. The distinction may be related to knowledge of the enzyme systems involved. It seems possible that the oxalacetic system may include unknown cofactors and that if these were available they could be used to obtain activity in water homogenates. Data of Utter, Wood and Reiner (42) and observations in our own laboratory suggest that homogenization in water leads to the release of enzymes such as DPN-ase so that inactivation of the phosphorylated cofactors proceeds more rapidly in the water homogenates than in the isotonic homogenates. In instances in which the cofactor can be supplied and its hydrolytic enzyme can be inhibited, as in the malic system, the water homogenate may be used and may actually be preferable. In systems that are not fully understood, such as the oxalacetic and phosphorylative systems, the KCl homogenate must be used for the present. In any event, the nature of the media must be specified for each system, thus we refer to "water homogenates" and alkaline isotonic KCl or "AIK homogenates."

The AIK homogenates seem to present numerous advantages over previously used media when dealing with labile systems. The KCl may be superior to a complete or balanced medium which contains phosphate, calcium, magnesium and other ions which act as activators for various enzyme systems, but this has not been adequately tested. The AIK homogenates are relatively free from agglutination and seem to give a high yield of broken cells on the basis of the numbers of free nuclei, although this is difficult to establish. The earlier method of measuring cytolysis (25) has been found to be based on a property of the subcellular particles rather than of the whole cells.

That further improvements in homogenization media are imminent is foreshadowed by the work of Hogeboom, Schneider and Pallade (6), who reported that the mitochondria obtained from water or isotonic homogenates were spherical and believed to be products of the rod-shaped mitochondria visible in cells. By use of hypertonic media such as 30 per cent sucrose they were able to obtain mitochondria which had the intracellular morphology. Liver homogenates prepared with 30 per cent sucrose gave succinoxidase values approximating those generally obtained with water. Studies on the more labile systems have not been reported.

The AIK homogenates are made with isotonic KCl (1.15 per cent) to which enough $KHCO_3$ is added to give a pH of 7.7–8.1 when in equilibrium with air. We add 8 ml of 0.04M $KHCO_3$ per liter of KCl and aerate for 20–30 min.

6. *Enzyme analysis.*—The goal of the homogenate technique is to arrange the experimental conditions so that the measured activity of the enzyme is a measure of the concentration of one particular enzyme. This is attained only when certain conditions are fulfilled. The first requirement is that irrelevant reactions do not confuse the measurement, which in most cases is either O_2 uptake or CO_2 output as measured in a Warburg microrespirometer. When a given reaction is being studied in

and, in effect, isolate the reaction while leaving the enzyme in a complex milieu.

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taken; in fact, occasionally normal values are used from experiments done at a different time and perhaps under somewhat different conditions. That this practice is not advisable is evident, since there sometimes is unexplained variation in activity from one day to the next even with the same enzyme preparation.

Assuming that a straight line function is obtained with increasing concentration of homogenate in both normal and diseased tissue, there still is a possibility of error in the comparison. An example may serve to illustrate the point.

Racker and Krimsky (31) showed that sodium ion inhibits anaerobic glycolysis by brain. Nevertheless Utter *et al.* (41) showed that straight line functions could be obtained even though sodium ions were used in the reaction mixture. Obviously if there were sufficient difference in the sodium content of normal and of infected tissue, there would be a difference in the glycolysis, and it would be judged that there was a difference in enzyme content of normal and of infected tissue. Actually the possibility of error is remote on the basis of sodium ion because the concentration required for inhibition is fairly high. Nevertheless it is not improbable that an unknown enzyme inhibitor which was effective in high dilution could produce such an effect.

It would seem advisable to heat-inactivate the preparations from abnormal tissue and add the heated preparation to homogenates of normal tissue to determine whether any inhibitors or activators are present in the heated sample. In a similar manner there could be checks between heated normal and abnormal homogenates and also between heated normal and normal, and heated abnormal and abnormal, homogenates. This procedure would provide an additional check for detection of unknown factors along with the dilution technique used by Dr. Potter. The procedure would by no means be infallible because the inhibitors might be heat labile.

Whereas in principle, at least, it is undoubtedly true that homogenates should be used for the quantitative measurement of enzymes and that extraction procedures should be avoided, in actual application difficulty is frequently encountered in use of homogenates, especially if the system is complex. The destruction of necessary cofactors or enzymes by enzymes in the homogenate may lead to more variation in activity than would result from incomplete recovery of the enzyme during partial purification procedures.

It all sums up to the fact that a good assay can be done when the reaction is simple, does not involve many cofactors and the system is well understood; but assays are very difficult in other circumstances.

THE HOMOGENIZER

1. *Construction.*—The homogenizer consists of a pestle which rotates inside a close-fitting test tube (28). The test tube is an ordinary 16×150 mm pyrex tube. The pestle is made to fit the average size tube. It is made of heavy-walled glass tubing having an outer diameter slightly larger than the inside diameter of the average test tube, so that when the tube and pestle have been ground with carborundum a perfect fit can be obtained. The glass tubing is sealed at one end and blown to a shape approximating the inside of the bottom of the test tube. It is then constricted at a point about 30 mm from the sealed end, pulled off and sealed to a 6 mm glass rod about 220 mm long. The cylindrical bulb should then have straight sides over a distance of about 20 mm. The pestle is made

terms of O_2 uptake it is important that the other sources of O_2 uptake have been eliminated. This is accomplished as outlined in the foregoing sections 3 and 4.

The second requirement is that the given enzyme be the limiting factor in the reaction. This can be tested by adding each of the known components of the system until further addition gives no increase in rate. Obviously each addition has to be made with all the other components present in adequate amount. When the given enzyme is the rate-controlling factor, the rate of the reaction (i.e., O_2 uptake or CO_2 output per time interval) is proportional to the amount of homogenate added, and this test can frequently be made a part of the routine assay method. The basis of the assumption that under the conditions described the activity is a measure of the enzyme concentration is the following:

Let E = enzyme, S = substrate, A = activator, i.e., coenzyme or prosthetic group, P = product.

$$\begin{aligned} E + A &\rightleftharpoons EA \\ EA + S &\rightleftharpoons EAS \\ EAS &\rightleftharpoons EAP \\ EAP &\rightleftharpoons EA + P. \end{aligned}$$

The enzyme forms a complex with the substrate and the activator, indicated by EAS. If all the other enzymatic components are in excess, the conversion of EAS to EAP is proportional to the amount of EAS. The problem is to have as little of E, EA and EAP as possible, with all of the enzyme in the form of the active enzyme-substrate complex. From the law of mass action the addition of excess A and S will tend to convert all of the E into EAS at the beginning, when none of the product is present, but as P accumulates, the amount of EAP becomes greater and the reaction slows down.

Thus if the rate is constant and proportional to the amount of homogenate, it is likely to be a valid measure of the amount of enzyme present.

Comment by H. G. Wood

It cannot be overemphasized that all of the suggestions and the implications of each step in the assay given here should be followed. The problem is difficult, and especially so when an attempt is made to compare normal and abnormal or diseased tissue as to their enzyme content. This is one of the most important applications of the technique, and it should be clearly realized that there are several sources of error in the comparison.

Each step should be tested on both the normal and the abnormal tissue. It does not necessarily follow that a straight line function with increase in homogenate concentration will be obtained with diseased tissue in the same conditions in which normal tissue will give a straight line. Another important point is to use the same conditions rigidly throughout for assay of the diseased and normal tissue. For example, if it is necessary to use aseptic technique with the diseased tissue, the controls should be treated in the same way so that time relationships, etc., are identical in both tests. Often these precautions have not been

pestle is attached to the chuck of a cone-driven stirring motor of the Cenco or Sargent type and driven at maximal speed (about 1000 rpm). The friction-drive stirrer is desirable because if a pestle sticks the friction drive slips instead of breaking the pestle or tube (see Fig. 1).

To prepare a tissue homogenate, a tissue sample weighing 500–1000 mg is carefully weighed on a torsion balance of 1 g capacity, graduated in fifths of a milligram, such as that made by the Roller-Smith Company. The tube and pestle are kept in a beaker of ice and water with 1 ml of the homogenization medium already in the tube to facilitate transfer of the tissue to the bottom of the tube. The volume of the medium to be added is then calculated by multiplying the weight of the tissue by 9 and subtracting the volume of fluid in the tube. The remaining fluid is then added, and the tissue is ready to be homogenized. Formerly the homogenization was done in a small volume of fluid, but more recent knowledge regarding the hazards of acid production makes it desirable to carry out the homogenization in as large a volume as possible. With close-fitting homogenizers it is possible to effect thorough homogenization in the final volume, while avoiding local acidity as much as possible. Small shreds of connective tissue usually remain in the homogenate, and for this reason Mohr pipets with drawn-out tips and enlarged openings are used to pipet homogenates. Tissues such as heart and skeletal muscle cannot be conveniently homogenized without preliminary mincing with a scissors. These tissues are first weighed and then minced on a small piece of plate glass in the cold. The mince can then be transferred to a homogenizer tube and homogenization completed.

PREPARATIONS

The homogenate technique is based on the reconstruction of enzyme systems following dilution of the enzymes that are found in the homogenized tissue. To restore the enzymes to their full activity, the various coenzymes that are found in the original tissue have to be added back to compensate for the dilution and destruction that occurred in the diluted homogenate. These factors include cytochrome c, adenosine triphosphate (ATP), the pyridine nucleotides, cocarboxylase and the various phosphorylated intermediates and intermediary metabolites that may be used as substrates.

Almost none of these compounds are available as synthetic chemicals and must therefore be prepared from natural sources. The method of preparation will not be given here. The method for cytochrome c preparation as modified by Keilin and Hartree (7) has been found advantageous in this laboratory. LePage has given detailed instructions for the preparation of ATP (cf. 39) and DPN (9), and the latter has been placed on the market by Schwartz Laboratories, Inc., New York, who have also made a number of the other compounds available.

more effective by sealing 6 or 7 small beads about 2 mm in diameter to the bottom of the bulb. The beads are ground down on emery cloth until they have flat surfaces that approximate the inside of the test tube and have a right-angled edge which aids in cutting the tissue. The side walls of the pestle are then ground in a tube containing fine carborundum in water.

It is advisable to prepare several pestles and tubes at the same time, since the tubes have to be ground with a pestle that is too small to grind

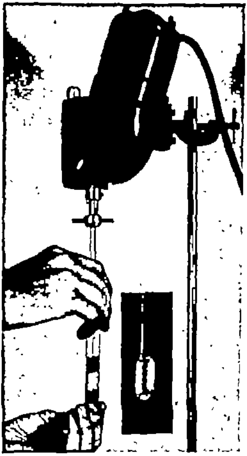

FIG. 1.—Apparatus for preparation of tissue homogenates. Insert shows pestle alone. The motor is a friction-drive stirrer.

tissue, and the pestles have to be ground in tubes that are too large to use for grinding tissue. After a number of pestles and tubes have been given ground glass surfaces they can be matched for use. The pestle should fit the tube as closely as possible without sticking. With continual use the tubes become somewhat loose and should be matched with other pestles.*

2. *Manipulation.*—In order to grind tissues rapidly and effectively, the

* The pestles have been placed on the market by the Central Scientific Company, Chicago. A modification that employs a conical tube is marketed by Rascher & Betzold, Inc., Chicago.

It was found that the cytochrome oxidase activity was highest in the water homogenates, and a correction factor was suggested to compensate for the incompleteness of homogenization, but at present it is uncertain whether this correction can be applied to the cytochrome oxidase system. It is important that the homogenization be very thorough.

Various investigators have been unable to obtain low rates of autoxidation in this system, and this has frequently been traced to metallic contaminants in the cytochrome preparation or in the other solutions employed. Tests in the complete system minus homogenate help to check this point.

2. *Succinic dehydrogenase.*—Assay for this enzyme, described by Schneider and Potter (35), depends on the presence of cytochrome oxidase in the tissue sample. If an excess of the oxidase is present and an excess of

TABLE 2.—SUCCINIC DEHYDROGENASE ASSAY*

	FLASK 1	FLASK 2
Addition	ml	ml
H_2O (to make 3 ml)	1.0	0.9
0.1M phosphate pH 7.4 (Table 1)	1.0	1.0
0.5M Na succinate pH 7.4	0.3	0.3
$1 \times 10^{-4}M$ cytochrome c	0.4	0.4
0.012M $CaCl_2$	0.1	0.1
0.012M $AlCl_3$	0.1	0.1
5% water homogenate	0.1	0.2
Results	μl O_2	μl O_2
O_2 uptake/10 min (ex.: rat liver, av. of 4 10-min periods)	20.0	42.0
O_2 uptake/10 min/mg wet weight; % dry weight = 30	4.0	4.2
Av. Q_{O_2} (μl of O_2/mg dry weight/hr)	80	84

* Conditions as in Table 1. Warburg flasks without side-arms may be used. Center cups contain 0.1 ml of 2N NaOH plus 1 sq cm folded filter paper; 10 min equilibration; temperature 37 C.

cytochrome c is added, the rate of O_2 uptake will depend on the rate of cytochrome c reduction. With succinate as the sole substrate, cytochrome reduction is brought about by succinic dehydrogenase (or by a combination of succinic dehydrogenase and a cytochrome c reductase that is closely bound to the dehydrogenase (*cf.* 37)). The chief problem is prevention of oxalacetate formation or accumulation, since this compound inhibits succinic dehydrogenase. It was found that the addition of both calcium and aluminum ions resulted in O_2 uptake that was proportional to tissue concentration (35), and these ions are therefore included in the reaction mixture given in Table 2. The calcium ions prevent oxalacetate formation (see p. 275). The nature of the aluminum effect is not fully understood (35).

3. *Malic dehydrogenase.*—The assay for this enzyme using the homogenate technique was described by Potter in 1946 (26). Chief precautions are prevention of oxalacetate accumulation and prevention of DPN de-

INDIVIDUAL ENZYMES

1. *Cytochrome oxidase.*—An improved method for assay of this enzyme was described by Schneider and Potter in 1943 (35). The assay is carried out on 1 per cent water homogenates of tissues. It is sometimes convenient to prepare a 10 per cent homogenate in order to achieve better sampling, and then to dilute to 1 per cent for use. The assay depends on a nonenzymatic reduction of cytochrome c, which then serves as the substrate for cytochrome oxidase. The assay is complicated by the fact that any compound that will reduce cytochrome c nonenzymatically is also more or less auto-oxidizable, and any assay for cytochrome oxidase must be corrected for O_2 uptake that results from spontaneous oxidation of the reductant of the cytochrome. The auto-oxidation of the ascorbic acid in the absence of tissue may be higher than in the presence of tissue if critical amounts of metal contaminants are present. Potter and Schneider therefore devised a three point assay that enables one to correct for the rate of auto-oxidation in the presence of the tissue. The three points are obtained by measuring the rate of O_2 uptake at three different concentrations of tissue that are chosen to give O_2 uptake rates of 20–60 cu mm of O_2/10 min. The amount of tissue to use depends on the tissue under study and must be determined by trial. In the case of rat liver the appropriate amount was 0.10, 0.15 and 0.20 ml of 1 per cent homogenate (Table 1). The tissue samples were not chilled and the reaction mixtures were set up at room temperature. The three flasks per sample are set up as in Table 1.

The ascorbate is prepared just before the test by adding 0.1N NaOH to solid ascorbic acid in the proportion of 1 ml of the alkali per 20 mg of the acid.

TABLE 1.—CYTOCHROME OXIDASE ASSAY*

	FLASK 1	FLASK 2	FLASK 3
Addition	ml	ml	ml
H_2O (to make 3 ml)	0.30	0.25	0.20
0.1M phosphate (NaH_2PO_4–NaOH to pH 7.4)	1.00	1.00	1.00
2.4×10^{-4}M cytochrome c	1.00	1.00	1.00
4×10^{-3}M $AlCl_3$	0.30	0.30	0.30
0.114M Na ascorbate; pH 6.7–7.0	0.30	0.30	0.30
1% water homogenate	0.10	0.15	0.20
Results	$\mu l\ O_2$	$\mu l\ O_2$	$\mu l\ O_2$
O_2 uptake/10 min (ex.: rat liver, av. of 4 10-min periods)	30.4	43.2	56.0
Subtract extrapolation to zero tissue concentration	4.8	4.8	4.8
Corrected uptake/10 min	25.6	38.4	51.2
O_2 uptake/10 min/mg wet weight; % dry weight = 30	25.6	25.6	25.6
Q_{O_2} (μl of O_2/mg dry weight/hr)	512	512	512

* Warburg flasks without side-arms may be used. Center cups contain 0.1 ml of 2N NaOH plus 1 sq cm folded filter paper; 10 min equilibration; temperature 37 C.

the rate is proportional to the tissue concentration. The rate of inorganic phosphate formation must be corrected for the amount of inorganic phosphate in the tissue and in the ATP. The reaction mixture is kept in a small volume in order to conserve ATP. The reaction mixtures are set up as in Table 4.

TABLE 4.—ADENOSINE TRIPHOSPHATASE ASSAY*

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Addition	ml	ml	ml	ml	ml
H_2O (to make 0.65 ml)	0.25	0.15	0.50	0.30	0.35
0.5M diethylbarbiturate pH 7.4	0.15	0.15	0.15	0.15	0.15
0.04M $CaCl_2$	0.05	0.05	0.05	0.05	0.05
0.01M ATP pH 7.4	0.20	0.20	.	.	0.20
1% water homogenate added 2 or 3 min after tubes are placed in thermostat	0.10	0.20	...	0.20	..
Results of P analyses	1 mg liver	2 mg liver	Reagents	Tissue	ATP
Optical density (E)	0.336	0.553	0	0.018	0.119
Subtract ATP blank	0.119	0.119			
	0.217	0.431			
Subtract tissue blank	0.009	0.018			
Corrected E values	0.208	0.416			
E/mg liver/15 min	0.52	0.52			
γP/mg liver/15 min	13.1	13.1			

* Reaction mixtures in 10 × 50 mm test tubes; incubation for 15 min at 37 C; reaction stopped with 0.1 ml of 50% trichloroacetic acid; 0.3 ml aliquots used for P analyses

Comment by H. G. Wood

It seems possible that this assay may be subject to error under certain conditions. First, ATP may be formed by synthetic reactions and in part mask the concurrent breakdown of ATP. The ATP synthesis might not occur unless ATP was added to start the reactions, for example in glycolysis. However, in DuBois and Potter's assay the high dilution of homogenate and lack of energy source would make unlikely the occurrence of such synthesis. The second and perhaps more probable source of error involves the role of a hexosephosphatase. If hexokinase were present in the homogenate and also a small amount of glucose or hexosephosphate, ATP would phosphorylate the glucose and the resulting hexosephosphate would be broken down by the hexosephosphatase, yielding inorganic phosphate. In this case the phosphate of ATP would be converted to inorganic phosphate without intervention of ATP-ase. The following reactions are illustrative:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose-6-}PO_4 + \text{ADP}$$

$$\text{glucose-6-}PO_4 \xrightarrow{\text{hexosephosphatase}} \text{glucose} + H_3PO_4$$

$$\text{Sum} = \quad \text{ATP} \longrightarrow \text{ADP} + H_3PO_4$$

Whereas these reactions may not greatly influence the assay of ATP-ase, they are possible sources of error and should be borne in mind.

struction. Neither of these ends can be completely attained, but they are probably effected to an extent that permits an estimation of the malic dehydrogenase. The oxalacetate is removed nonoxidatively by means of a transamination reaction (glutamate + oxalacetate → ketoglutarate + aspartate) and the DPN destruction is blocked by nicotinamide. It is probably unnecessary to add more transaminase because of some parallelism between the amount of malic dehydrogenase and transaminase; however, a good method for preparing transaminase is available (34) and it can be added to the reaction mixture if necessary

As in the case of cytochrome oxidase and succinic dehydrogenase it is probably unnecessary *to keep the homogenate and the reaction mixture* cold during the preliminary operations. Recent studies (30) have indicated that delayed addition of DPN from a side-arm is probably superior to the use of iced flasks with no side-arm.

The reaction mixture employed is given in Table 3.

TABLE 3.—MALIC DEHYDROGENASE ASSAY*

	FLASK 1	FLASK 2
Addition	ml	ml
H_2O (to make 3 ml)	0.7	0.6
0.1M phosphate pH 7.4 (Table 1)	0.8	0.8
0.5M Na glutamate	0.3	0.3
0.5M nicotinamide	0.3	0.3
1% DPN (equivalent to 6000 γ/ml) in side-arm	0.2	0.2
4×10^{-4}M cytochrome c	0.3	0.3
5% water homogenate	0.1	0.2
Results	μl O_2	μl O_2
O_2 uptake/10 min (ex.: rat liver, best 2 consecutive 5 min periods in 1st 20 min)	25.0	48.8
O_2 uptake/10 min/mg wet weight	5.0	4.88
Q_{O_2}	100	97.6

* Warburg flasks with one side-arm may be used; folded filter paper; 10 min equilibration; temperature 37 C; all acidic substances neutralized to pH 7.4–7.6.

4. *DPN—cytochrome c reductase.*—Assay for this enzyme may be carried out using the reaction mixture employed in the malic dehydrogenase assay (Table 3), except that an excess of malic dehydrogenase is added to make the cytochrome reductase the limiting factor in the oxidation (26). An excellent preparation of malic dehydrogenase has been described by Straub (38); it may be lyophilized or frozen in solution. It is used at a level of about 50 γ per flask (30). In general, the level of cytochrome reductase is slightly higher than that of malic dehydrogenase.

5. *ATP-ase.*—The rate at which ATP can be dephosphorylated may be studied by the method of DuBois and Potter (4). Water homogenates prepared at room temperature may be used. Homogenization should be very thorough. *The rate of inorganic phosphate formation* is measured under conditions such that the reaction is linear with respect to time and

The oxalacetic oxidase system has given good results with liver, in which the rate of O_2 uptake has been proportional to the amount of homogenate, and oxidation of oxalacetate has been much more rapid than that of pyruvate alone. Studies with other tissues indicate that the system is still not fully reconstructed.

2. *Oxidative phosphorylation.*—Studies have been carried out with whole homogenates following the principles established by experiments with extracts, particularly those of Ochoa (20, 21). In earlier work with homogenates it was assumed that water homogenates would have to be used in order to obtain complete cell disruption and overcome permeability barriers against ATP (23, 24, 27). It was subsequently found that the isotonic KCl homogenate was relatively cell-free and that it permitted oxidation of oxalacetate whereas water homogenates, of liver at least, were inactive on oxalacetate (27). The water homogenate permits succinate and malate oxidation and may be useful in studying phosphorylations connected with single oxidative steps. However, water homogenization appears to lead to marked phosphatase action, and fluoride must be included in the reaction system to show a net uptake of inorganic phosphate. Furthermore, accumulation of the high energy phosphate presents a problem that has not been solved. When inorganic phosphate uptake occurs the phosphate can be accumulated in the form of ATP, using ADP or AMP as the phosphate acceptor. However, breakdown of ATP to inorganic phosphate increases with ATP concentration, and the ideal maneuver would be to transfer the high energy phosphate to a secondary reservoir so as to maintain the ATP concentration low. Such a secondary reservoir exists in the form of the creatine-phosphocreatine system and was utilized by Potter (24) to capture the energy of oxidation. It was shown that the creatine transphosphorylase was low in liver tissue and that additions of the crude enzyme increased the uptake of inorganic phosphate. However, this transphosphorylase has not been obtained in pure form, and use of the crude enzyme is not recommended.

Another method of accumulating high energy phosphate outside the ATP reservoir would be to utilize the hexokinase and phosphofructokinase reactions, accumulating the phosphate in the form of hexosephosphate. This maneuver is fraught with difficulties, however, for several reasons. The factors which govern activity of the hexokinase and phosphofructokinase systems are poorly understood and they seem to exhibit a low order of activity in certain tissue homogenates. However, yeast hexokinase could be added were it not for a second hazard, namely, the uncontrolled action of the phosphatases that split off the phosphate from the hexosephosphates. The third complication is the fact that the hexosediphosphate that might be formed will be glycolyzed if the reaction mixture contains DPN. Glycolysis would introduce another source of phosphate esterification and would also lead to secondary reactions. Thus the ingenious interlocking of the metabolic cycles makes difficult the technical separation of the component systems.

6. *Miscellaneous.*—The homogenate technique can obviously be employed for assay of any single enzyme that catalyzes a reaction in which the substrate and cofactors are readily obtainable. If the enzyme acts directly on the substrate and produces a measurable end-result without the co-ordinated action of other enzymes, the problem is relatively simple. Numerous hydrolytic reactions fall in this category. The main difficulty is control of side reactions, and each case represents a special problem. Many of the enzymes concerned in vital processes are not readily studied individually, but at the present state of knowledge can be studied as members of an enzyme system, discussed in the following section.

ENZYME SYSTEMS

1. *Oxalacetic oxidase system.*—Work on this system is still in the preliminary stages, and the system here described cannot be said to be an assay for a specific enzyme. It is apparently the system referred to by Green (5) as "cyclophorase" in that it catalyzes the oxidation of all the members of the Krebs cycle. Since the oxidation of oxalacetic acid seems to be a more critical step than some of the others, we have concentrated on the use of oxalacetic acid as the substrate. Oxidation of oxalacetic acid may be studied in homogenates if certain precautions are taken. In contrast to the succinic and malic dehydrogenases, this enzyme system is rendered ineffective by homogenization in water and must be kept cold until the assay is begun. This may be a reflection of the inadequacy of present knowledge of the system. The oxidation probably follows a partial conversion to pyruvic acid and the Krebs condensation. The reaction mixture is given in Table 5.

TABLE 5.—ASSAY OF THE OXALACETIC OXIDASE SYSTEM*

	FLASK 1	FLASK 2
Addition	ml	ml
H_2O	1.2	1.2
0.5M KCl	0.4	0.4
0.1M $MgCl_2$	0.1	0.1
0.1M K phosphate (KH_2PO_4 + KOH to pH 7.4)	0.1	0.1
4.0×10^{-4}M cytochrome c	0.1	0.1
0.01M K-ATP	0.3	0.3
1.15% KCl	0.3	0.1
0.0267M oxalacetic acid + 0.02M K_2CO_3, aerated	0.3	0.3
10% AIK homogenate	0.2	0.4
Results	µl O_2	µl O_2
O_2 uptake (ex.: rat liver, best 2 consecutive 10 min periods)	42.8	82.8
O_2 uptake/20 min/mg wet weight	2.14	2.07
Q_{O_2} (µl of O_2/mg dry weight/hr)	21.4	20.7

* Warburg flasks without side-arms may be used. Center cups contain 0.2 ml of 2N NaOH plus 1 sq cm of folded filter paper; 10 min equilibration; temperature 37 C; flasks in ice until homogenate is added; homogenates prepared in cold alkaline isotonic KCl.

enate (42). These results were in contrast to those of Meyerhof and Geliaskowa (13), who found high activity in extracts and low activity in whole homogenates, but the discrepancy was apparently due to the fact that Meyerhof was using less HDP and the main substrate was glucose. When both glucose and HDP were present in higher concentrations, results were more comparable to those of Utter *et al.* (12, 14). The extracts were shown to contain very little ATP-ase, while the residues contained about 90 per cent of this enzyme.

Comment by H. G. Wood

Although differences in HDP concentration probably account for the fact that Utter *et al.* obtained higher activity with homogenates than did Meyerhof and Geliaskowa, there is an additional discrepancy in that the extracts obtained by Utter *et al.* with isotonic saline were of low activity, whereas Meyerhof and Geliaskowa's extracts were very active. The most likely explanation is that the grinding was not as effective in the experiments of Utter *et al.* or that there was a difference in the efficiency of centrifugation in preparation of the extract.

The reaction mixture described by Utter *et al.* has been the model for similar studies on chick embryo (18) and on tumor homogenates (19) by Novikoff, Potter and LePage, on liver by Reiner (33), on kidney, liver, brain and tumor by LePage (10) and on brain by Racker and Krimsky (31, 32). The reaction mixture described in Table 6 is based on the experience in this laboratory (10, 18, 19) following the other publications.

TABLE 6.—ASSAY OF THE GLYCOLYTIC SYSTEM (WITHOUT FLUORIDE)*

ADDITION	FINAL MOLARITY	FLASK 1	FLASK 2
		ml	ml
0.154M KCl	0.051†	0.8	0.7
0.1M potassium phosphate pH 7.6	0.01	0.3	0.3
0.5M $KHCO_3$	0.05	0.3	0.3
0.01M K-ATP	0.00067	0.2	0.2
0.4M nicotinamide	0.04	0.3	0.3
0.01M K-DPN	0.00033	0.1	0.1
0.04M K-HDP	0.004	0.3	0.3
0.1M glucose	0.01	0.3	0.3
0.1M $MgCl_2$	0.0033	0.1	0.1
0.015M K-pyruvate	0.005	0.1	0.1
10% AIK homogenate	...	0.2	0.3

*Warburg flasks without side-arms may be used; 6 min temperature equilibration; temperature 37 C; gas phase 95% N_2–5% CO_2 (Linde O_2 free); flasks gassed by alternate evacuation and refilling with gas.

† Including KCl in homogenate. In the fluoride system, final molarity of fluoride is 0.01, and the pyruvate is increased to 0.01M.

The rate of glycolysis in this system is considerably faster than that of glycolysis in slices. This difference was attributed (10) to the fact that glycolysis in slices is based on glucose as a substrate, whereas in the homogenate the hexosediphosphate is the main substrate. It was suggested that in the slices the rate is probably determined by the rate of

At present the creatine-phosphocreatine system appears to be the most promising as an accumulator of phosphate bond energy, although it cannot be used in the case of liver until pure creatine transphosphorylase is available. Among the other advantages of the creatine-phosphocreatine system is the fact that phosphocreatine is readily separated from ATP and inorganic phosphate. This is done by means of a scaled down application (23) of the method of Fiske and Subbarow.

Comment by H. G. Wood

It is to be noted that hexosephosphate has been successfully used as a reservoir of organic phosphate by Ochoa and Cori and others. In the tissues which they used the hexosephosphatase activity was not high. Also, there is a possible difficulty in the use of creatine as the phosphate acceptor in that the reaction catalyzed by creatine transphosphorylase is reversible, so that in the presence of ATP-ase the high energy phosphate would be lost through conversion of creatine phosphate to ATP and destruction of ATP by ATP-ase.

Systems for study of oxidative phosphorylation in homogenates have been published (23, 24, 27), but it cannot be said that a routine technique has been established, and consequently none is presented here.

3. *Fatty acid oxidase system.*—Oxidation of fatty acids by washed liver homogenates was observed by Munoz and Leloir (15) and by Lehninger (8), who showed the importance of ATP in this system. Potter (25) confirmed this work and emphasized the effect of tonicity on the system, showing that hypotonic media during either the assay or the washing process caused marked decreases in activity. The assay has not been placed on a quantitative basis, but it is now clear that the procedures could be readily developed on the basis of published data (8, 25). The essential point is use of the isotonic homogenate and use of sufficient centrifugal force to throw down all of the fatty acid oxidase during the washing period; this might be checked by measuring the amount of succinoxidase in the residue (25). This succinoxidase value could probably be raised to nearly 100 per cent of the value for the whole homogenate and could be used to indicate recovery of the particles in the residue.

4. *Glycolytic enzymes.*—The first attempt to study glycolysis in fortified tissue homogenates was described by Utter, Reiner and Wood (41). Their discussion is a most lucid analysis of the role of in vitro measurements in the study of metabolic disorders. They obtained high rates of glycolysis in brain homogenates over periods of an hour or more. Their reaction mixture contained ATP, glucose, HDP, DPN, Mg ions, phosphate, nicotinamide and bicarbonate, in addition to brain homogenate. They later compared homogenates with extracts obtained as the supernates from centrifuged homogenates and found that in the case of isotonic saline homogenates the extract contained much less activity than the whole homogenate, whereas in the case of water homogenates the extract contained about the same amount of activity as the whole homog-

enzyme system required for the synthesis and in the absence of the enzymes that catalyze the breakdown of the energy-rich intermediates. A fourth approach that might be included is addition of ATP at a rate that is greater than the sum of the multiple dephosphorylating mechanisms, but this is not to be recommended since the accumulation of products of ATP breakdown is likely to affect the reaction adversely.

The various successful syntheses carried out thus far have succeeded at least partially in attaining the conditions classified above, and although routine studies on diverse materials are not yet indicated, a number of studies show the possibility of studying the synthetic reactions. The syntheses of acetylcholine (16, 17), urea (2) and peptide bond models such as hippuric acid (1), p-aminohippuric acid (3), glutamine (36) and acetylated aromatic amines (11) all attest the importance of the ATP reservoir in biosynthesis. Further progress in controlling the multiple pathways of ATP breakdown should make it possible to study many other syntheses.

CONCLUSION

The methods outlined in the foregoing discussion are primarily concerned with attempts to determine the concentration or potential activity of various enzyme systems in animal tissues. It is realized that the activity of the individual enzyme systems is subject to many controlling factors in vivo. These factors involve not only spatial segregation of enzymes but changes in the chemical environment. The availability of quantitative methods prepares the way for study of the control mechanisms, because the distribution of enzymes in tissues in different physiologic states, in different parts of the tissue and in different parts of the cell can be determined. Furthermore, it is possible to study the manner in which enzymes produce changes in the chemical environment and the effect of these changes on the activity of other enzymes.

Thus the measurements of enzyme concentration are the first stage of the larger task, which is to discover the control mechanisms and mode of organization of the enzymes in the living animal, so that derangements in metabolism may be understood and corrected.

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conversion of glucose to HDP, whereas in the homogenate the rate is determined by the enzymes that occur between HDP and lactate.

The reaction mixture can be modified slightly to include fluoride, which blocks conversion of phosphopyruvate to pyruvate by preventing the formation of 2-phosphoglycerate. The fluoride inhibits phosphatase and ATP-ase to a considerable extent, so that a net uptake of inorganic phosphate can occur as a result of the extra phosphate fixed by oxidation of triosephosphate. This phosphate must be transmitted via the ATP system to glucose or creatine if a net uptake is to occur. Since formation of pyruvate is prevented, extra pyruvate must be added to act as an H acceptor for oxidation of triosephosphate to phosphoglycerate. In this system, according to theory, the CO_2 due to acid, the phosphoglycerate and the lactate should all be formed in equivalent amounts.

Comment by H. G. Wood

When complex systems such as those described in section 4 are used, it is apparent that the measured rate of the over-all reaction is not an indication of the activity of the individual enzymes. The slowest reaction of the group will determine the over-all rate. The value of this method of enzyme study is considerably increased if it is combined with an analysis of end-products and intermediary compounds, such as phosphate esters. In this way a disturbance in the balance of enzyme reactions can be detected and an indication of the alteration in an individual enzyme may be deduced. An enzyme assay for the single enzyme can then be undertaken.

It is, of course, much more difficult to make comparative measurements with these complicated systems because the over-all reaction is a delicate balance of many linked reactions.

NOTE.—Methods for determining the end-products and intermediates are given in the following contribution by Dr. LePage.

5. *Synthetic reactions.*—The studies directed toward the elaboration of oxidative or glycolytic systems that would maintain the ATP reservoir have been carried out on the assumption that this reservoir is of considerable importance as a source of energy for the various synthetic reactions that occur in cells. A number of studies in recent years have indicated the fulfilment of this premise, and it is now fairly clear that any enzyme system in which ATP is nonspecifically dephosphorylated is rather unlikely to support a synthetic reaction. Even in the event of syntheses that involve other energy sources it seems likely that the ATP reservoir is in dynamic equilibrium with the system and that both will be depleted together. Thus an in vitro enzymatic synthesis must be carried out by (*a*) eliminating all or most of the mechanisms for the dephosphorylation of ATP except the particular synthetic reaction under study, (*b*) providing energy-yielding reactions such as glycolysis or oxidation that will maintain the ATP reservoir in the presence of multiple dephosphorylating mechanisms, (*c*) preparing energy-rich intermediates that will react exergonically to give the synthesis in the presence of the

ANALYSES FOR TISSUE METABOLITES WITH IN SITU FREEZING TECHNIQUES

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In studies of cellular metabolism it is frequently advantageous to measure the reactants and products of the enzymatic reactions in vivo. To carry out valid analyses for metabolites, there is needed a means of rapidly inactivating enzymes, or suspending their activity, without affecting the metabolites chemically. Many analytical data in the literature have suffered from insufficient attention to the necessity for rapid stopping of enzymatic reactions.

A number of investigators (11, 21, 47) have recognized the necessity for using rapid freezing techniques to suspend tissue reactions in study of carbohydrate metabolism, where it is readily observed how rapidly autolytic changes modify the levels of reactants. It is clearly demonstrated, for example, that removal of a rat brain and inactivation in trichloracetic acid, a process which can be accomplished in a few seconds, results in phosphocreatine analyses which are only 25–30 per cent of those obtained by first freezing the intact animal in liquid air, dissecting the brain out in the frozen state, powdering the sample and then inactivating the frozen powder in trichloracetic acid. Similarly, one finds a several-fold higher lactic acid level in the former instance.

Several freezing agents are available, each of which has its advantages. In working with certain accessible tissues such as muscle, it is sometimes possible to achieve excellent results by packing the area with *dry ice.* This does not give especially rapid freezing but minimizes stimulation. Tissues may be immersed in mixtures of dry ice with organic solvents (alcohol, ether). Here a temperature gradient of some 108 C is available and heat transfer is rapid because of the good conductivity of the organic liquid. However, if small samples are used, there is some disadvantage in that the sample becomes wet with the solvent, and this type of mixture is not particularly convenient for chilling vessels in which tissues are to be powdered. A better gradient has been obtained by the use of isopentane chilled with liquid nitrogen or liquid air. Extra precautions are required when *liquid air* is used with organic solvents because the combination gives dangerously explosive mixtures. Liquid air itself is a good agent for freezing. It can be poured over a tissue, such as the exposed brain of an anesthetized animal, or tissues may be immersed in it.

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PREPARATION AND EXTRACTION OF TISSUE

The tissue, or whole animal if desired, is dropped in liquid air. With whole animals it is advisable to use an anesthetic; study of various agents indicated that nembutal was quite suitable. Study of several tissues of the same rat can be done by this means. If the animal is frozen as soon as it reaches surgical anesthesia (3–4 min), little effect of the anesthetic on the tissues can be noted; it merely prevents a convulsive response to the sudden cold contact. That low lactate levels, etc., obtained with nembutal on resting tissues are not due just to inhibition of glycolysis is indicated by the facts that high lactate levels are obtained in brain with nembutal overdosage and that tumors, which have no innervation, give identical results when frozen with or without nembutal anesthesia (30). Tissues are dissected out of a carcass in the frozen state with chisel and hammer. Because of cleavage lines, which can be learned by experience, it is thus possible to obtain kidney, brain and heart intact and free of other tissues. Samples of muscle and liver are readily obtained. Precautions must be observed to avoid including small splinters of bone with brain and muscle samples.

Insertion of a thermocouple down the esophagus to the stomach of a 300 g rat and immersion of the rat in liquid air permitted determination of the rate of freezing of internal organs. For the internal organs to reach 0 C, some 20 sec was required. To reach liquid air temperature 40–60 sec was required. For this reason some sacrifice of resting conditions is made for the liver samples by freezing the whole animal. Opening the animal and excising into liquid air appears to give optimal results for liver. This is not necessary for other tissues, in our experience.

When dissection is complete, the tissue sample (1–2 g) is put in the steel grinder (Fig. 1) with liquid air. The grinder and pestle are chilled by use of liquid air, and the sample is hammered once or twice with the pestle just after the liquid air boils off. Any sample clinging to the pestle is quickly scraped off with a chilled spatula and more liquid air added to the resulting tissue powder. When the last of the liquid air boils off again, while the sample is still very cold, it is transferred to a small paper and quickly slid into a weighed tube containing 6–7 ml of 10 per cent trichloracetic acid. This inactivates the enzymes and prevents enzymatic change. Reweighing gives tissue weight. The sample is mixed briefly with a *loose* stainless steel or glass homogenizing pestle (40). This serves to mix and aid in extraction of the sample. Real homogenization of the tissue should be avoided, since interfering phosphorus compounds are added to the extract from liver by this procedure, and in brain samples homog-

There is the possible disadvantage that heat transfer is slowed by formation of a gaseous layer next to the sample. However, it provides an excellent temperature gradient (223 C) and is completely innocuous, does not contaminate the sample in any way and is quite safe as long as no contact with organic solvents is permitted and no attempt made to confine it to a closed system. Small animals such as mice and rats can be thrown directly into a vacuum bottle containing liquid air* and rapidly frozen. It is with the description of this technique and subsequent analysis of tissues for intermediates of carbohydrate metabolism that this discussion is mainly concerned.

That phosphorylated carbohydrate intermediates and related compounds play an indispensable role in intermediary metabolism of animal and certain other tissues is well established (32, 41). A great variety of analytical techniques have become available for measurement of the individual phosphorylated compounds in tissues. But since Lohmann's work (34) few attempts have been made to provide a co-ordinated scheme of methods. Yet if one wishes to study phosphorylation in intact tissues, especially on small experimental animals, a co-ordinated scheme of micromethods is essential. The components in which one is interested in such a study include inorganic phosphate (ortho-), total acid-soluble phosphorus, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenylic acid (AA), fructose-1,6-diphosphate (hexosediphosphate), glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, phosphoglyceric acid, phosphopyruvic acid, triosephosphates (glyceraldehyde phosphate and dihydroxyacetone phosphate), glycogen, lactic acid and coenzymes (pyridine nucleotides). Contemporary concepts of the interrelations of these materials have been reviewed by Lipmann (32), Kalckar (18), Burk (7), Potter (41) and others.

The method described here permits estimation of each of the materials listed in the preceding paragraph on a relatively small sample of tissue (1–2 g wet weight). It has proved satisfactory for muscle, heart, brain, kidney and liver (1, 28, 29) and is useful, with limitations and modifications, for bacteria, algae, oat seedlings and yeast (2, 12, 25–27).

The method consists, in brief, in extraction of the "acid-soluble" phosphorus from the powdered frozen tissue with trichloracetic acid, separation of this extract into three well defined fractions by means of barium and alcohol and determination of the known components of these fractions by means of their characteristic properties. When this has been accomplished, the results are checked by construction of a balance sheet to determine how much of the phosphorus of each fraction is accounted for in terms of the components measured.

It must be recognized that in new experimental circumstances, or in other tissues, modifications in the procedure may be necessary. It contains, as now designed, no provision for estimation of the phosphogluconic

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ATP, ADP, hexosediphosphate, phosphoglyceric acid and a part (20–30 per cent) of the hexose esters. The last are apparently carried down as double salts and can be removed by the following procedure. The precipitate is dissolved in 2–3 ml of 0.05N HCl, more barium acetate is added, and precipitation at pH 8.2 is repeated. The supernatant is poured off and added to the first one. The precipitate is suspended in 3–5 ml of 0.05N H_2SO_4 and the $BaSO_4$ centrifuged. The clear supernatant is neutralized and made to a known volume for analysis. This is referred to as the *barium-insoluble fraction*. To determine its constituents, the following analyses are necessary: inorganic phosphorus, total phosphorus, phosphorus hydrolyzed in 7 min and 180 min (1N HCl, 100 C), ribose and fructose. It may also be examined for absorption at 260 mμ in a spectrophotometer.

The supernatant from the barium precipitations is treated with 4 volumes of 95 per cent ethanol and readjusted (if necessary) to pH 8.2. The resulting precipitate is centrifuged at least 15 min in the cold and the supernatant drained off well. The precipitate is taken up in 3–5 ml of 0.05N H_2SO_4 and the $BaSO_4$ centrifuged. The supernatant is neutralized and made to a known volume for analysis. This is referred to as the *barium-soluble alcohol-insoluble fraction*. To determine its constituents the following analyses are made: inorganic phosphorus, phosphocreatine, ribose, fructose, reducing sugar, phosphorus hydrolyzed in 7 min (1N HCl, 100 C), nitrogen and nicotinic acid (spectrophotometric methods can be substituted for the last two).

The supernatant from the barium and alcohol precipitation contains a relatively small proportion of the acid-soluble phosphorus (5–20 per cent, varying with the different tissues) and practically none of the esters concerned in the phosphorylative glycolysis system of Embden-Meyerhof (trace of pyridine nucleotides). This *barium-soluble alcohol-soluble fraction* is usually ignored in the analysis. However, it contains any aminoethyl phosphate (39, 8) and propanediol phosphate (30, 31) that was present in the sample. To study these, the fraction is evaporated at 30–40 C under reduced pressure to approximately 20 per cent of its original volume, when most of the alcohol has been removed. It is then treated with H_2SO_4 to a final concentration of 0.4N. The $BaSO_4$ is centrifuged off and the supernatant extracted twice with diethyl ether to remove the trichloracetic acid and certain colored impurities. No phosphorus enters the ether layer. The water layer is evaporated almost to dryness under reduced pressure and taken up in 95 per cent ethanol. Neutralization with KOH leaves salts behind and gives a solution containing the phosphorus esters. On addition of inorganic phosphate and uranium, any aminoethyl phosphate precipitates. Addition of basic lead acetate precipitates any propanediol phosphate present.

Procedure B.—The foregoing procedure is adequate for brain, kidney, heart and muscle because the cold trichloracetic acid extract does not contain any of the tissue glycogen (glycogen can be estimated on residue).

enization leads to suspension in the extract of colloidal material which prevents precipitations.† The tissue residue is centrifuged and the supernatant poured off. A second extraction is made with 5–6 ml of 5 per cent trichloracetic acid and this added to the first.

Whether traces of phosphatases go into solution and survive the trichloracetic acid and what factors may be concerned is not known, but the results are most reliable when the procedure is carried through rapidly and most of the analyses are finished the same day. Once frozen, samples keep indefinitely in a deepfreeze at −20 C or below, permitting preparation of samples on one occasion and analysis on another.[1]

FRACTIONATION WITH BARIUM

Aliquots of the trichloracetic acid extracts are withdrawn, either before or after neutralization (volumes recorded), and used for analysis for

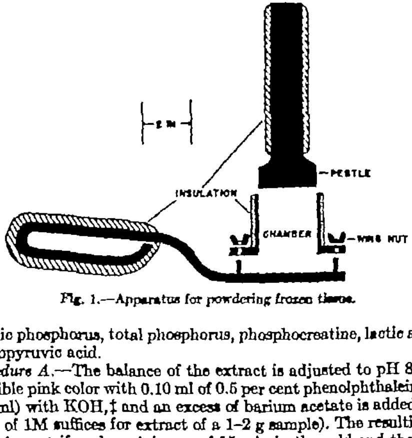

Fig. 1.—Apparatus for powdering frozen tissue.

inorganic phosphorus, total phosphorus, phosphocreatine, lactic acid and phosphopyruvic acid.

Procedure A.—The balance of the extract is adjusted to pH 8.2 (just discernible pink color with 0.10 ml of 0.5 per cent phenolphthalein added per 10 ml) with KOH,‡ and an excess of barium acetate is added (0.25–0.35 ml of 1M suffices for extract of a 1–2 g sample). The resulting precipitate is centrifuged a minimum of 15 min in the cold and the supernatant poured off. The precipitate contains the inorganic phosphorus,

† This material is brought down by use of alcohol and higher pH values, as in the procedure of Stone (48), but separation of the esters is thereby destroyed; e.g., fructose-6-phosphate comes down with hexosediphosphate.

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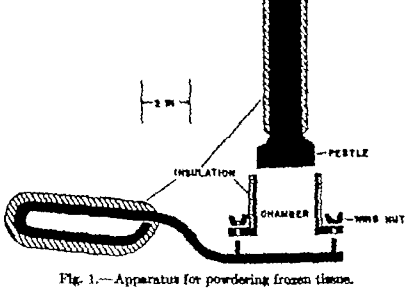

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However, in extraction of liver (and certain primary tumors) with cold trichloracetic acid a large part of the glycogen goes into the extract. Since this glycogen as well as that in the tissue residue is to be measured and since the glycogen in solution prevents barium fractionation by keeping the insoluble salts from precipitating, a means of separating it out

TABLE 1.—SOME CHEMICAL PROPERTIES OF THE PHOSPHORYLATED ESTERS AND RELATED COMPOUNDS

Fraction	Compound	Method of Determination	% Hydrolysis* in 1N HCl at 100 C: 7 Min	% Hydrolysis* in 1N HCl at 100 C: 180 Min	Reducing Values to Folin-Malmros Method
Barium-insoluble	ATP	ATP, pentose	66	89	0
	ADP	ATP, pentose	50	79	0
	Hexosediphosphate	Fructose	20.5	...	9.5
	3-Phosphoglyceric acid	Resistant to 3 hr hydrolysis (corrected)	0	2	0
Barium-soluble alcohol-insoluble	Glucose-1-phosphate	Phosphorus and reducing sugar hydrolysed in 7 min.	100	...	0 (66.4% after hydrolysis)†
	Glucose-6-phosphate	Reducing sugar	...	10.5	13.2
	Fructose-6-phosphate	Reducing sugar, fructose	...	74	31.8
	Phosphopyruvic acid	Phosphorus released by alkaline iodine	46	100	0
	Triosephosphate	Alkaline hydrolysis	46	100	...
	Adenylic acid	260 μ absorption spectrum	...	58.7	0
	Phosphopyridine nucleotides	Nicotinic acid, reduced form—340 μ absorption spectrum	...	58.7	0
	Phosphocreatine	Inorganic phosphorus not precipitated with calcium	100	...	0
	Ribose-5-phosphate	Pentose	.	58.7	19.7
	Inosinic acid	Pentose, 248–290 μ absorption spectrum, conversion to uric acid	...	58.7	0
Barium-soluble alcohol-soluble	1,2-Propanediol phosphate	Lead precipitation after removal of interfering factors	.	1.7	0
	Aminoethyl phosphate	Precipitation with inorganic phosphorus and uranium	...	...	0
Glycogen	Glycogen	Reduction after acid hydrolysis	...	100	0

* Hydrolysis rates of resistant esters are affected to some extent by the level of inorganic phosphorus present; those of easily hydrolyzable esters are not so affected.
† 69.2 per cent theoretical, 66.4 per cent found.

without loss of any esters is required. This can be accomplished by the following modification of procedure A.

The trichloracetic acid extract, unneutralized, is treated with an equal volume of 95 per cent ethanol. The glycogen precipitates and is centrifuged off immediately. The supernatant, which still contains all the esters, is neutralized to pH 8.2 and barium added as in procedure A; then sufficient 95 per cent ethanol is added to make a total of 4 volumes

Fig. 2.—Methods of Extraction and Fractionation.

Extraction:

Frozen, powdered tissue projected into tube containing cold 10% trichloracetic acid, centrifuge (0–5 C throughout)

Residue: re-extract with 5% trichloracetic acid | Extract | Combine extracts and neutralize (unless to be used for Proc. B).

Residue: used to determine acid-insoluble phosphorus and glycogen | Extract

Procedure A: Neutralized extract, pH 8.2: add large excess of barium acetate, chill, centrifuge in cold

Precipitate dissolved in 0.1N HCl, add excess of barium acetate solution, adjust pH to 8.2 with KOH, chill, centrifuge

Precipitate: dissolve in 0.05N H_2SO_4, centrifuge → Add 4 vol neutralized (pH 8.2) 95% ethanol, chill, centrifuge

$BaSO_4$: wash with small volume 0.05N H_2SO_4 | Neutralize to pH 7.0 and make to some definite vol. *Barium-insoluble fraction*

$BaSO_4$: discard

Dissolve in 0.05N H_2SO_4, centrifuge | Concentrate under reduced pressure, remove barium and make very acid with H_2SO_4, extract twice with ether, evaporate further under reduced pressure. *Barium-soluble alcohol-soluble fraction*

$BaSO_4$: wash with 0.05N H_2SO_4 | Neutralize to pH 7.0 and make to some definite vol. *Barium-soluble alcohol-insoluble fraction*

$BaSO_4$: discard

Procedure B: Unneutralized trichloracetic acid extracts: treat with equal volume of 95% ethanol in cold, centrifuge

Precipitate: Extractable glycogen of the tissue; no esters | Supernatant—neutralize to pH 8.2 with KOH, add large excess of barium acetate and add more 95% ethanol to make 4 vol, chill, centrifuge

Precipitate: dissolve in 0.1N HCl, neutralize to pH 8.2 with KOH, add excess barium acetate, chill, centrifuge | *Barium-soluble alcohol-soluble fraction:* treat as in Proc. A

Precipitate: dissolve in 0.1N HCl, add excess barium acetate, neutralize to pH 8.2, chill, centrifuge

Barium-insoluble fraction: remove barium and neutralize as in Proc. A → *Barium-soluble alcohol-insoluble fraction:* remove barium with excess H_2SO_4, neutralize as in Proc. A

The precipitate is centrifuged, the supernatant discarded and the precipitate washed with a small volume of a 1:5 dilution of the $CaCl_2$ reagent. The inorganic phosphorus is in the precipitate. The phosphocreatine is discarded in the supernatant. The washed precipitate is dissolved in dilute HCl and made to the original volume of the sample, then inorganic phosphorus is determined. This value is presumably the "true" inorganic phosphorus of the tissue. Although ATP hydrolyzes in alkaline solutions (48), it does not measurably contribute to the inorganic phosphorus in this brief period. Other more labile esters such as acetyl phosphate may appear as inorganic phosphorus. In liver, where phosphocreatine is supposedly absent (15), a value is obtained which would indicate some 250 μM of phosphocreatine per 100 g. This may be esters of fatty acids or other labile materials not precipitable with calcium.[3]

3. *Total phosphorus.*—The sample is digested with 0.15 ml of 8N H_2SO_4 in an oven or sand bath for 30–60 min at 130–160 C, removed, partially cooled (this can be carried out in the pyrex colorimeter tubes) and 1–2 drops of 30 per cent hydrogen peroxide added. The tube is replaced in the oven for 15–20 min, then partially cooled, and 1 ml of water added to the residue. This diluted sample is now heated at 100 C for 10 min to decompose pyrophosphates. The cooling before additions avoids causing the acid to fume, which is undesirable because some phosphorus may be lost. Now inorganic phosphorus is determined without further addition of acid.

4. *Lactic acid.*—This is patterned after the method of Barker and Summerson (6). The sample, containing 3–30 μg of lactic acid, is pipetted into a clean 16 × 150 mm test tube, made to 4.5 ml, and 0.5 ml of 20 per cent $CuSO_4 \cdot 5H_2O$ added. Approximately 0.5 g of $Ca(OH)_2$ is added and dispersed by shaking. The copper-calcium hydroxide precipitate removes interfering materials. The precipitate is redispersed several times in the course of 30 min or more, then centrifuged. A 0.50 ml aliquot of the supernatant is transferred to a clean tube, with care not to include any of the precipitate. Tube and sample are chilled in an ice bath and 3.0 ml of concentrated H_2SO_4 is added slowly from a pipet or buret, with vigorous shaking. This precaution, cooling and shaking, avoids localized heating which can cause irregular results (further oxidation of the acetaldehyde to acetic acid). Tubes are heated in a boiling water bath 5 min, cooled to below 30 C and 1 drop each of 4 per cent $CuSO_4 \cdot 5H_2O$ and p-hydroxydiphenyl reagent added. (By using the same dropper each time, including the standards, one need not measure these reagents except by drops.) The reagent is immediately dispersed by shaking. The tubes are incubated at 28–30 C for 30 min or more with occasional shaking to redisperse the reagent. Then the tubes are heated in a boiling water bath 90 sec, cooled and transferred to colorimeter tubes, and transmission is read against a reagent blank at 565 mμ.

[3] See also commentary, page 354.

per volume of water solution. The precipitate contains all the esters of the first two fractions described in procedure A. The supernatant is the *barium-soluble alcohol-soluble fraction* and is treated as in procedure A. The precipitate is dissolved in 0.05N HCl (5–8 ml) and the pH adjusted to 8.2 with $Ba(OH)_2$§ (0.4N). The precipitate is centrifuged in the cold, reprecipitated, $BaSO_4$ removed and the solution neutralized as in procedure A. This is the *barium-insoluble fraction.* The supernatants from this precipitation are treated with excess H_2SO_4, $BaSO_4$ is centrifuged out and the solution neutralized. This is the *barium-soluble alcohol-insoluble fraction.* Each fraction is now analyzed for its constituents as indicated earlier.‖

BASIC ANALYTIC METHODS

The microcolorimetric methods are described as we employ them. Obviously these can be modified and others substituted at the convenience of the investigator. We use a Cenco-Sheard spectrophotometer, with sets of 13 × 100 mm tubes selected for identical transmission and graduated at 3.0 ml. This procedure has the advantage that colorimetric tests can all be accomplished in a 3.0 ml volume, with the result that ranges for the analytical methods are approximately 1.5–15 μg with accuracy of ±2 per cent. Accuracy can be increased by use of duplicate and triplicate analyses when material is available. The methods obey Beer's law over the ranges indicated.

1. *Inorganic (ortho-) phosphate, including phosphocreatine and other labile esters.*—This is patterned after the method of Fiske and Subbarow (13). The sample is mixed in a colorimeter tube with 0.15 ml of 8N H_2SO_4 (HCl can be used), 0.25 ml of 2.5 per cent ammonium molybdate and 0.15 ml of Fiske-Subbarow reducing agent (materials added in the order named). Volume is made to 3.0 ml with distilled water and color permitted to develop 8–10 min before reading. Almost maximal color is developed within 2 min (97 per cent) and further color development between 10 and 30 min is negligible, so that a series of tubes may be read. Light transmission is measured at 660 mμ.

To make the reducing *reagent*, to 250 mg of sodium 1-amino-2-naphthol-4-sulfonate add 97.5 ml of 15 per cent sodium bisulfite and 2.5 ml of 20 per cent sodium sulfite. Warm in a water bath to 40–50 C and stir rapidly until the solid has dissolved. The reagent is stable for several weeks if kept out of direct sunlight and well stoppered (to prevent loss of SO_2).

If phosphocreatine is present, incubation of 20 min should be permitted before addition of the reducing agent to permit full hydrolysis of the phosphocreatine.

2. *"True" inorganic phosphorus.*—The method is patterned after that of Fiske and Subbarow (14). To the neutralized sample, in a graduated tube, is added $^1/_4$ volume of 10 per cent $CaCl_2$ saturated with $Ca(OH)_2$.

§ This minimizes the addition of salts.
‖ See also commentary, page 353.

The precipitate is centrifuged, the supernatant discarded and the precipitate washed with a small volume of a 1:5 dilution of the $CaCl_2$ reagent. The inorganic phosphorus is in the precipitate. The phosphocreatine is discarded in the supernatant. The washed precipitate is dissolved in dilute HCl and made to the original volume of the sample, then inorganic phosphorus is determined. This value is presumably the "true" inorganic phosphorus of the tissue. Although ATP hydrolyzes in alkaline solutions (48), it does not measurably contribute to the inorganic phosphorus in this brief period. Other more labile esters such as acetyl phosphate may appear as inorganic phosphorus. In liver, where phosphocreatine is supposedly absent (15), a value is obtained which would indicate some 250 μM of phosphocreatine per 100 g. This may be esters of fatty acids or other labile materials not precipitable with calcium.[3]

3. *Total phosphorus.*—The sample is digested with 0.15 ml of 8N H_2SO_4 in an oven or sand bath for 30–60 min at 130–160 C, removed, partially cooled (this can be carried out in the pyrex colorimeter tubes) and 1–2 drops of 30 per cent hydrogen peroxide added. The tube is replaced in the oven for 15–20 min, then partially cooled, and 1 ml of water added to the residue. This diluted sample is now heated at 100 C for 10 min to decompose pyrophosphates. The cooling before additions avoids causing the acid to fume, which is undesirable because some phosphorus may be lost. Now inorganic phosphorus is determined without further addition of acid.

4. *Lactic acid.*—This is patterned after the method of Barker and Summerson (6). The sample, containing 3–30 μg of lactic acid, is pipetted into a clean 16 $\times$ 150 mm test tube, made to 4.5 ml, and 0.5 ml of 20 per cent $CuSO_4 \cdot 5H_2O$ added. Approximately 0.5 g of $Ca(OH)_2$ is added and dispersed by shaking. The copper-calcium hydroxide precipitate removes interfering materials. The precipitate is redispersed several times in the course of 30 min or more, then centrifuged. A 0.50 ml aliquot of the supernatant is transferred to a clean tube, with care not to include any of the precipitate. Tube and sample are chilled in an ice bath and 3.0 ml of concentrated H_2SO_4 is added slowly from a pipet or buret, with vigorous shaking. This precaution, cooling and shaking, avoids localized heating which can cause irregular results (further oxidation of the acetaldehyde to acetic acid). Tubes are heated in a boiling water bath 5 min, cooled to below 30 C and 1 drop each of 4 per cent $CuSO_4 \cdot 5H_2O$ and p-hydroxydiphenyl reagent added. (By using the same dropper each time, including the standards, one need not measure these reagents except by drops.) The reagent is immediately dispersed by shaking. The tubes are incubated at 28–30 C for 30 min or more with occasional shaking to redisperse the reagent. Then the tubes are heated in a boiling water bath 90 sec, cooled and transferred to colorimeter tubes, and transmission is read against a reagent blank at 565 mμ.

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[3] See also commentary, page 354.

A standard curve can be run with either pure zinc lactate or C.P. 85 per cent lactic acid. With the latter, the acid is diluted to approximately 1N, boiled 10 min to depolymerize, then titrated with standard alkali and phenolphthalein and diluted appropriately.

The sulfuric acid used should be a reagent grade, kept free of metals, organic matter and nitrates. Traces of nitric acid in it will destroy results (25). The *p-hydroxydiphenyl reagent* is made by dissolving it in 5 per cent NaOH and diluting to 1.5 per cent p-hydroxydiphenyl, 0.5 per cent NaOH. It will keep indefinitely in a refrigerator for several months at room temperature in a dark bottle.

A blank should always be run through the complete procedure to make sure that no contamination has occurred from glassware or reagents. Necessary precautions in this method include care that the apparatus is clean, since contact of fingers with the lip of a tube can add sufficient lactate to influence results seriously. Glassware cleaned in chromic acid should be rinsed and immersed in dilute alkali solution (0.2N NaOH) for a few minutes before final rinsing to insure removal of chromium. As soon as the operator becomes convinced of the necessity of the precautions outlined (especially cleanliness of glassware and slow addition of acid to sample in the cold), consistently accurate results are easily obtained.[4]

5. *Fructose.*—This is patterned after the method of Roe (45). The sample is made to 0.60 ml in a colorimeter tube. To it are added 0.60 ml of 0.1 per cent resorcinol in 95 per cent ethanol and 1.80 ml of 30 per cent HCl. The solutions are mixed and the sample is heated 8 min at 80 C. The tube is cooled and transmission determined against a reagent blank at 490 mμ. Range is 3–30 μg. The resorcinol reagent is stable for several months.

6. *Pentose.*—This is patterned after the method of Mejbaum (30). The sample is made to 1.5 ml in a colorimeter tube and mixed with 1.5 ml of orcinol reagent. The tube is heated 30 min in a boiling water bath, then cooled and read against a reagent blank at 660 mμ. Range is 1.5–15 μg. The method determines pentose in nucleotides, nucleosides and nucleic acids as well as in the free form. A standard of ribose, xylose or arabinose can be used, for all arrive at the same color intensity after 30 min heating, though at different rates. Concentrated HCl containing 0.1 per cent $FeCl_3$ is kept as a stock solution and the *orcinol reagent* made just before use by addition of 10 mg of orcinol per ml. Orcinol as usually obtained requires recrystallization from benzene.[5]

7. *Reducing sugar.*—This is patterned after the method of Folin and Malmros (16). The sample is made to 0.48 ml in a colorimeter tube. To it are added 0.24 ml of 0.40 per cent $K_3Fe(CN)_6$ and 0.12 ml of cyanide-carbonate reagent. The solutions are mixed, heated in a boiling water bath 8 min, cooled 1–2 min, and 0.60 ml of ferric iron reagent added.

[4] See also commentary, page 354.
[5] See also commentary, page 355.

The volume is made to 3.0 ml with distilled water and the color read against a reagent blank at 520 mμ. Range is 2–20 μg of glucose. It is necessary to have glass bubbles on the tops of the tubes to minimize reoxidation by air.

The *cyanide-carbonate reagent* is made by dissolving 8 g of anhydrous sodium carbonate in 40–50 ml of water, adding 15 ml of freshly prepared 1 per cent NaCN and diluting to 500 ml.

The *ferric iron reagent* is made by dissolving 20 g of gum ghatti in 1 liter of water, filtering and adding a solution of 5 g of $Fe_2(SO_4)_3$, 75 ml of 85 per cent H_3PO_4 and 100 ml of water. After mixing, about 15 ml of 1 per cent $KMnO_4$ is slowly added to destroy reducing materials present in the gum ghatti and the solution is allowed to stand several days before use. The reagent is stable indefinitely.

8. *Nitrogen.*—The sample is digested in a pyrex tube in oven or sand bath at 150–190 C, with 0.5 ml of 5N H_2SO_4 (containing 150 mg of copper selenite per liter), for 12 hr or more. Then 1–2 drops of 30 per cent hydrogen peroxide is added and the tube heated over a microburner until white fumes develop. The sample is cooled, washed into a colorimeter tube with small portions of water, 0.60 ml of 5.5N KOH added, mixed and 0.7 ml of modified Nessler's solution added. Volume is adjusted to 3.0 ml and the tube contents mixed. After 10 min transmission is measured at 520 mμ. Range is 5–25 μg. The precision is not as great as with other analyses, so it is well to have duplicates or triplicates.

To make up *Nessler's reagent*, 5.0 g of KI and 5–7 g of HgI_2 are ground together in a mortar with 50 ml of water and made to 500 ml. There should be an excess of HgI_2. Let stand overnight, then filter off excess HgI_2. To 500 ml of this filtrate add 160 ml of a 1.5 per cent gum ghatti solution. Mix and dilute to 1500 ml. This reagent is stable indefinitely.

9. *Nicotinic acid.*—This is patterned after the method of Bandier and Hald (5). The sample is mixed with an equal volume of 2N KOH and heated 1 hr in a boiling water bath (to hydrolyze coenzymes and nicotinamide). On cooling the sample is neutralized to pH 7.0 with 2N HCl and diluted to 1.35 ml and equilibrated in a water bath at 78–80 C. Then 0.15 ml of cyanogen bromide reagent is added and incubation continued 5 min. The sample is cooled to room temperature, mixed with an equal volume (1.5 ml) of photol‖ reagent and incubated 1 hr at room temperature, protected from direct light. Transmission is then measured at 420 mμ. Range is 1.5–12 μg.[¶]

The *cyanogen bromide reagent* is made by addition of 10 per cent KCN solution to a saturated solution of bromine in water until the solution is just decolorized. The *photol reagent* is a saturated water solution of photol made by shaking 3–4 g in 50 ml of water. Both reagents should be made up fresh within 1–2 hr of use.

‖ Monomethyl p-aminophenol sulfate.

¶ See also commentary, page 355.

A standard curve can be run with either pure zinc lactate or C.P. 85 per cent lactic acid. With the latter, the acid is diluted to approximately 1N, boiled 10 min to depolymerize, then titrated with standard alkali and phenolphthalein and diluted appropriately.

The sulfuric acid used should be a reagent grade, kept free of metals, organic matter and nitrates. Traces of nitric acid in it will destroy results (25). The *p-hydroxydiphenyl reagent* is made by dissolving it in 5 per cent NaOH and diluting to 1.5 per cent p-hydroxydiphenyl, 0.5 per cent NaOH. It will keep indefinitely in a refrigerator for several months at room temperature in a dark bottle.

A blank should always be run through the complete procedure to make sure that no contamination has occurred from glassware or reagents. Necessary precautions in this method include care that the apparatus is clean, since contact of fingers with the lip of a tube can add sufficient lactate to influence results seriously. Glassware cleaned in chromic acid should be rinsed and immersed in dilute alkali solution (0.2N NaOH) for a few minutes before final rinsing to insure removal of chromium. As soon as the operator becomes convinced of the necessity of the precautions outlined (especially cleanliness of glassware and slow addition of acid to sample in the cold), consistently accurate results are easily obtained.[4]

5. *Fructose.*—This is patterned after the method of Roe (45). The sample is made to 0.60 ml in a colorimeter tube. To it are added 0.60 ml of 0.1 per cent resorcinol in 95 per cent ethanol and 1.80 ml of 30 per cent HCl. The solutions are mixed and the sample is heated 8 min at 80 C. The tube is cooled and transmission determined against a reagent blank at 490 mμ. Range is 3–30 μg. The resorcinol reagent is stable for several months.

6. *Pentose.*—This is patterned after the method of Mejbaum (36). The sample is made to 1.5 ml in a colorimeter tube and mixed with 1.5 ml of orcinol reagent. The tube is heated 30 min in a boiling water bath, then cooled and read against a reagent blank at 660 mμ. Range is 1.5–15 μg. The method determines pentose in nucleotides, nucleosides and nucleic acids as well as in the free form. A standard of ribose, xylose or arabinose can be used, for all arrive at the same color intensity after 30 min heating, though at different rates. Concentrated HCl containing 0.1 per cent $FeCl_3$ is kept as a stock solution and the *orcinol reagent* made just before use by addition of 10 mg of orcinol per ml. Orcinol as usually obtained requires recrystallization from benzene.[5]

7. *Reducing sugar.*—This is patterned after the method of Folin and Malmros (16). The sample is made to 0.48 ml in a colorimeter tube. To it are added 0.24 ml of 0.40 per cent $K_3Fe(CN)_6$ and 0.12 ml of cyanide-carbonate reagent. The solutions are mixed, heated in a boiling water bath 8 min, cooled 1–2 min, and 0.60 ml of *ferric iron reagent* added.

[4] See also commentary, page 354.

[5] See also commentary, page 356.

with magnesia mixture in alkaline solution to remove most of the inorganic phosphorus, then carrying out measurement of total and 3 hr hydrolyzed phosphorus. Resistant phosphorus is now all phosphoglyceric phosphorus.

4. *Phosphocreatine.*—This is computed from the inorganic phosphorus determined on the original extract, and on the calcium precipitate, by difference. It is apparently an accurate representation in muscle, brain, heart, kidney and tumor, but questionable in the case of liver, as mentioned previously. The phosphocreatine appears in the barium-soluble alcohol-insoluble fraction.[7]

5. *Glucose-1-phosphate.*—This ester is easily hydrolyzable in acid and can be computed by measurement on the barium-soluble alcohol-insoluble fraction of either inorganic phosphorus or reducing sugar before and after hydrolysis (7 min, 1N HCl, 100 C) The two values usually agree well, that from reducing sugar tending to be slightly higher owing to slight hydrolysis of other esters. If phosphopyruvic acid or triosephosphate is present, a correction must be applied to the easily hydrolyzable phosphorus for a 46 per cent hydrolysis of these esters.[8]

6. *Fructose-6-phosphate.*—This can be computed from the fructose analyses on the barium-soluble alcohol-insoluble fraction, since it is the only fructose ester present in the fraction. It responds only 60.5 per cent of the theoretical to the fructose test, so that fructose analysis must be multiplied by the factor 2.39 to obtain fructose-6-phosphate, unless the pure ester is used for standard, in which case no factor would be necessary. It responds, without hydrolysis, to the reducing sugar test to an extent equivalent to 31.6 per cent of its weight of glucose. It is slowly hydrolyzed in acid, requiring 5 hr to hydrolyze 90 per cent in 1N HCl at 100 C.

7. *Glucose-6-phosphate.*—This ester is usually a major constituent of the barium-soluble alcohol-insoluble fraction. It has a reducing value equivalent to 13.2 per cent its weight of glucose and can be calculated from the reducing sugar measured on this fraction corrected for reduction due to fructose-6-phosphate and ribose phosphate not combined in nucleotides.

In certain specific circumstances errors may be caused in this measurement by glutathione and glucose. However, glutathione will not precipitate in this fraction unless it exceeds 1.2 mg/ml in the original extract, which is not a usual physiologic circumstance. If a tissue is hyperglycemic, as may be especially true of autolyzed liver for example, there is danger that the analysis for glucose-6-phosphate by this means will be high because of precipitation of part of the glucose with barium and alcohol. If this circumstance is met, it can be surmounted by taking an aliquot of the barium-soluble alcohol-insoluble fraction and reprecipitating it several times with barium and alcohol, redissolving in water each

[7] See also commentary, page 353.
[8] See also commentary, page 355.

DETERMINATION AND CALCULATION OF SPECIFIC COMPOUNDS

1. *Adenosine triphosphate (ATP) and adenosine diphosphate (ADP).*—Both of these compounds are quantitatively precipitated in the barium-insoluble fraction. If inorganic phosphate is absent, ADP becomes much more soluble, but in the presence of inorganic phosphorus the solubility is only 4 μg/ml. Both ATP and ADP contain adenine, ribose and phosphorus. They are the only pentose compounds in this fraction. ATP has two easily hydrolyzable phosphates; ADP has one. Consequently both can be calculated by measurement of ribose, inorganic and easily hydrolyzable phosphorus (7 min, 1N HCl, 100 C). For example, a molar ratio of easily hydrolyzable phosphorus to ribose of 1.85 indicates that 85 per cent of the ribose is present as ATP, 15 per cent as ADP. A check on the ribose analysis of the fraction can be provided by measurement of absorption of the solution at 260 mμ in the spectrophotometer (see adenylic acid, p. 350). This absorption is due to the adenine component.

2. *Fructose-1,6-diphosphate (HDP).*—In the presence of inorganic phosphate, this compound is quantitatively precipitated with barium. In the absence of inorganic phosphate, it is considerably more soluble (pH 8.2, excess barium, soluble to the extent of 2.8 mg/ml). Since it is the only fructose ester in the fraction, it can be estimated by measurement of fructose. As it does not react as the theoretical amount of fructose (52.5 per cent), fructose measured must be multiplied by the factor 3.60 to convert it to HDP present. If pure HDP is used as standard, no factor is necessary. Since it is the only reducing compound present in the fraction¶ (9.5 per cent that of an equal weight of glucose), the measurement can be checked by measurement of reducing sugar.

Little HDP is normally found in intact tissues. Consequently it is a small part of the phosphorus in the fraction. If any appreciable amount is found, the easily hydrolyzable phosphorus attributable to ATP and ADP needs to be corrected for a 26.5 per cent hydrolysis of the HDP phosphorus.

3. *Phosphoglyceric acid.*—This compound (3-phosphoglyceric) is usually a measurable component of the tissue phosphorus. It is quantitatively precipitated with barium. Although a colorimetric method is available for its measurement (42), it has generally proved unsatisfactory owing to interference of other materials. The ester can be determined by making use of its resistance to acid hydrolysis. From the inorganic phosphorus after 3 hr of hydrolysis (1N HCl, 100 C) and the total phosphorus of the fraction, a figure can be computed for "resistant phosphorus." This includes the phosphoglyceric acid phosphorus, but must be corrected for unhydrolyzed phosphorus of ATP and ADP (41.3 per cent of the stable phosphate). This test may be refined, if desired, by treating an aliquot of the barium-insoluble fraction with mercury in acid solution to remove the nucleotides (phosphoglyceric soluble) and

¶ Glutathione is soluble to the extent of >2 mg/ml under these conditions and will not occur in this fraction.

pH 8.2 and the ribose phosphate precipitated as before with barium and alcohol. The adenine remains in solution. The precipitate is dissolved, barium removed and nitrogen analyses are carried out on it and the original fraction. The difference, nitrogen lost on hydrolysis, is the adenine nitrogen. Hence adenylic acid can be calculated.

Both methods require a correction for any adenylic acid present as phosphopyridine nucleotides (coenzymes I and II—DPN and TPN). These are determined separately.

11. *Coenzymes.*—The phosphopyridine nucleotide coenzymes are not completely recovered here (some go into the barium-soluble alcohol-soluble fraction). The amount of the two (sum) that is present can be determined by measurement of nicotinic acid, which is a component of both, and calculation from this analysis.

With a Beckman spectrophotometer or similar instrument, a second method is available—measurement of absorption of the reduced co-enzymes at 340 mμ. Because of the reproducibility of results with this instrument, the range can be carried down to 5 μg (computed as DPN, since there is usually several times more DPN than TPN) with a ± 5 per cent error. It is more accurate when higher levels are used. For this assay, two 0.50 ml aliquots of the barium-soluble alcohol-insoluble fraction (containing 5–175 μg of DPN) are pipeted into 13 $\times$ 100 mm tubes, 0.40 ml of fresh 2 per cent $NaHCO_3$ is added to each and 0.10 ml of water to one, 0.10 ml of a fresh 3 per cent solution of sodium hydrosulfite in 1 per cent $NaHCO_3$ to the other. Both are incubated 20 min at room temperature, then 3 ml of 1 per cent $NaHCO_3$–1 per cent Na_2CO_3 solution added to each and a stream of air passed through each for 5 min. Both solutions are used to measure transmission at 340 mμ against a blank of the $NaHCO_3$–Na_2CO_3 buffer. Some absorption due to other materials is obtained in the unreduced sample. This is subtracted from the absorption obtained with the reduced sample. The difference is absorption due to reduced phosphopyridine nucleotides. The figures so obtained agree with nicotinic acid assays within limits of error.

12. *Ribose phosphate.*—There is in some tissues an excess of ribose, measured in the barium-soluble alcohol-insoluble fraction, above and beyond that accounted for by adenylic acid and phosphopyridine nucleotides. This appears to be free ribose phosphate. It can be determined by difference, using the ribose, adenylic acid and phosphopyridine nucleotide analyses of the fraction. The significance of this component, whether it is present as a product of nucleotide metabolism or of oxidative hexose degradation, is not known.

13. *Glycogen.*—Except for tissues like liver, cold trichloracetic acid does not extract glycogen, and it can be measured on the tissue residue left after the esters are extracted. This tissue residue is mixed with 5 ml of 30 per cent KOH and heated in a boiling water bath until completely dissolved (10–30 min), then treated with 6 ml of 95 per cent ethanol while hot, cooled and the glycogen precipitate centrifuged out. This is

time, all in the same tube. By this procedure it was found that approximately 85 per cent of the glucose was lost on each precipitation and as many as four such precipitations resulted in no measurable loss of glucose-6-phosphate (some of the other esters are not so well recovered). Reducing sugar measurements on this material, corrected for fructose-6-phosphate, are free from errors caused by interfering materials.

8. *Phosphopyruvic acid.*—This compound, when present, occurs in the barium-soluble alcohol-insoluble fraction. It is best to determine it on the original extract first. Its measurement is based on its property of being hydrolyzed by alkaline iodine to yield inorganic phosphorus (35). The determination is made by diluting the sample to 1.35 ml in a colorimeter tube, adding 0.1 ml of 2N KOH and 0.05 ml of 0.1M iodine in KI, incubating 15 min, then adding acid and discharging the excess iodine with sodium bisulfite. Inorganic phosphorus is measured and compared with that obtained without alkaline hydrolysis. The difference is computed as phosphopyruvic acid phosphorus. (Iodoform formation is ordinarily not sufficient to interfere.) This compound does not usually accumulate to a measurable extent in animal tissues.[9]

9. *Triosephosphate (dihydroxyacetone phosphate and 3-glyceraldehyde phosphate).*—These are determined by measuring alkali-labile phosphorus (37). Phosphopyruvic acid is not hydrolyzed in this test. The sample is mixed in a colorimeter tube with an equal volume of 2N KOH, incubated 20 min at room temperature, neutralized and inorganic phosphorus determined. The inorganic phosphorus hydrolyzed in this test on the barium-soluble alcohol-insoluble fraction is a measure of triosephosphate. Methods of differentiating the two have been described by Utter and Werkman (49). These esters do not ordinarily accumulate to a measurable extent in animal tissues.[10]

10. *Adenylic acid.*—When the necessary equipment is available (Beckman quartz spectrophotometer), adenylic acid is most simply estimated on the barium-soluble alcohol-insoluble fraction, in which it occurs, by measurement of absorption at 260 mμ in the spectrophotometer. Measurement of pentose sugars gives preliminary indication of the concentration. The aliquot of the fraction is diluted in M/15 sodium phosphate buffer at pH 7.0 and transmission read against a buffer blank. Range is approximately 2–20 μg of adenylic acid per ml (requires 3–4 ml). A standard curve can be constructed with adenylic acid or adenosine.

When the aforementioned equipment is not available, a second method may be used which has been demonstrated to give the same results with animal tissues. This method makes use of the property of adenylic acid by which all the adenine can be hydrolyzed by 1N HCl at 100 C in 10 min without any appreciable hydrolysis of the ribose phosphate component. This hydrolysis is carried out on an aliquot of the barium-soluble alcohol-insoluble fraction, with the hydrolyzed material neutralized to

[9] See also commentary, page 355.
[10] See also commentary, page 355.

explanation that enzymes of ATP utilization were failing more rapidly than enzymes of ATP synthesis is plausible. In the former case it is possible that the virus behaves in the same manner as an anesthetic, since no anesthetic was used on the controls (28).

The techniques described here provide a means of evaluating the in vivo mechanisms of ATP synthesis and also the mechanisms of ATP utilization, provided the correlated processes of function and ATP synthesis (glycolysis and respiration) can be simultaneously appraised (cf. 50).

Comment by William E. Stone

Preparation and extraction of tissue.—Dr. LePage has given an excellent description of this procedure. The writer would like to underscore the emphasis on the necessity of freezing in situ or use of an equivalent technique in the preparation of tissues for analysis.

When a sufficient weight of sample is available, preparation of the extract may be somewhat simplified by using only a single extraction. In calculating the dilution the tissue water is then included.

If facilities for centrifuging in the cold are not available, filtration in the refrigerator is a satisfactory substitute.

Fractionation with barium.—The writer's experience with the method is limited to study of cerebral tissue, a material which presents special difficulties. Behavior of the nucleotides is of particular concern. Before Dr. LePage's method became available a procedure was developed which has more limited scope and depends on fractional precipitation of calcium salts (48). Results obtained by this method have been considered reliable because the findings on normal and postmortem dog brain are in agreement with those of Kerr (23), who studied the nucleotides by quite different methods. The obvious discrepancy between the results on dogs by the calcium method and those on rats by the barium method (25) called for further investigation.

With procedure A, using the low concentration of barium acetate originally specified (25), it was found that only 5-10 per cent of the ribonucleotide was precipitated from extracts of normal dog brain, despite the fact that the nucleotide is known to consist largely of ATP (23). Only a similarly small fraction of the inorganic phosphate was precipitated. With procedure B, however, precipitation was greatly improved; approximately two thirds of the ATP and half the inorganic phosphate were carried down (as compared with the calcium method on the same filtrate). In the analysis by procedure B the conditions were approximately the same as with procedure A except that preliminary precipitation with barium and 4 volumes of ethanol preceded the fractionation. Thus it seems that preliminary precipitation permitted partial removal in the centrifugate of some constituent of the brain extract which tends to hold the nucleotides and inorganic phosphate in solution. What this substance may be can only be conjectured at present. It does not appear to be a colloidal material resulting from excessive grinding or homogenization of the tissue, since the results were the same when the coarsely crushed tissue was extracted with 10 per cent trichloracetic acid, homogenization being omitted. It seems possible that this finding may be related to observations of Meyerhof (38), who concluded that

patterned after the method of Good *et al.* (17). The supernatant is discarded and the precipitate hydrolyzed 2 hr in 1N HCl at 100 C. The glycogen obtained from the trichloroacetic acid extract of liver is hydrolyzed also. The hydrolysate is neutralized and reducing sugar measured with a copper reagent, such as that of Shaffer and Somogyi (46). The factor 0.927 can be used to convert glucose measured to anhydrous glycogen. This procedure is satisfactory for liver, kidney, heart and muscle, but another feature must be added in the analysis of brain. For this, the tissue residue is dissolved in KOH and treated with alcohol as before. The precipitate obtained is suspended in a warm mixture of chloroform-methanol (20 volumes of $CHCl_3$ + 80 volumes of methanol) and centrifuged. This is repeated twice before the hydrolysis in acid. The washing removes cerebrosides which otherwise cause error by hydrolyzing in acid to yield reducing compounds (22).[11]

14. *Aminoethyl phosphate.*—This compound is found in autolyzing tissues, probably from breakdown of sphingosine. If present, it appears in the barium-soluble alcohol-soluble fraction, where, as previously mentioned, it can be precipitated with uranium and inorganic phosphate provided the fraction is reduced to a low volume. It can be identified by elemental analysis.

15. *Propanediol phosphate.*—This compound is present in the barium-soluble alcohol-soluble fraction of tissues. It appears to constitute 5 per cent of the acid-soluble organic phosphorus of brain, 1–2 per cent of that of liver, kidney and tumor. Solubility of the uranium salt is unaffected by inorganic phosphorus (exceeds 4 mg/ml in solubility). It can be precipitated from this fraction when the volume is sufficiently reduced (solubility in presence of excess basic lead acetate is 0.13 mg/ml) by basic lead. It is highly resistant to alkaline or acid hydrolysis.

DISCUSSION

These methods and the properties of the compounds have been tested by use of the pure esters. In most instances they have also been confirmed by isolation of the compounds, from larger samples of tissue, in high yields (40–85 per cent). For tabulated analyses of animal tissues made with these methods, the reader is referred to reports in the literature (1, 28, 29, 30).

The in vivo level of the high energy phosphate reservoirs in a tissue always depends on the balance between synthesis and utilization. As has been pointed out (29), it is possible to have high energy phosphorus reservoirs remain in a tissue because of failure or blocking of the enzymes of utilization. It has been reported that brains infected with a virus had higher levels of ATP than did control brains (17a). In another study higher phosphocreatine levels were observed in failing hearts than were found in the functioning control hearts (50). In both instances the

[11] See also commentary, page 355.

It is essential that the temperature be carefully controlled during the first few minutes of color development. Maximal color intensity is attained if the tubes are cooled in ice water to 0 C before the p-hydroxydiphenyl is added and are held at this temperature for 15–30 min. They can then be placed in water at room temperature or above for about 30 min, after which final heating may be unnecessary since the solutions will be clear unless an excessive amount of p-hydroxydiphenyl has been used. It is advisable to include standard solutions with every series of determinations.

Pentose.—This procedure may be improved by reducing the $FeCl_3$ concentration, which should be 0.4 mM during the heating. At this concentration maximal color intensity is attained and the reaction is insensitive to variations in HCl concentration between 6N and 8N. With the higher concentration of $FeCl_3$ the reaction is very sensitive to variations in HCl and $FeCl_3$ concentrations. The time of heating may be reduced if HCl concentration is increased. It is necessary to avoid contamination from cork stoppers and similar materials.

In the writer's experience ATP and adenylic acid have given identical results and are suitable standards for the determination of nucleotides. The free pentoses, however, have given less color intensity per mole (after an extended heating time) with the reagent of Mejbaum as well as with the modified reagent. Since this result is contrary to the observations of Dr. LePage and of Albaum and Umbreit (3), it is suggested that it be checked by others using the method.

Nicotinic acid.—It would be advisable to buffer the solution by adding 0.15 ml of 10 per cent KH_2PO_4 before dilution to 1.35 ml and addition of cyanogen bromide (10).

Phosphocreatine.—When this is determined in the presence of ATP a slight error is introduced owing to slow hydrolysis of ATP during the time allowed for the hydrolysis of phosphocreatine. The error is minimized by limiting this period to not more than 30 min. Molybdate must not be added before the acid.

Glucose-1-phosphate.—If this is measured by the increase in reducing powder on acid hydrolysis, presence of adenylic acid would necessitate correction since its hydrolysis frees the reducing group of ribose phosphate. Determination of this substance might well be checked by the procedure of Kosterlitz and Ritchie (24).

Phosphopyruvic acid.—When phosphocreatine is present, sufficient time must be allowed for its hydrolysis after addition of molybdate. Since phosphopyruvate occurs only in small amounts, this measurement in most tissues represents a small difference between two relatively large values and can scarcely be relied on to establish the presence of phosphopyruvate. When ATP is present its hydrolysis is a further potential source of error.

A promising alternative procedure has been introduced by Conway and Hingerty (9), who determine phosphopyruvate in the barium-soluble alcohol-insoluble fraction by conversion to pyruvate and assay of the latter with the aid of 2,4-dinitrophenylhydrazine.

Triosephosphate.—The foregoing remarks on phosphopyruvate are equally applicable to the method for triosephosphate.

Conway and Hingerty (9) determine triosephosphate in the barium-soluble alcohol-insoluble fraction by conversion to phosphoglycerate, which is assayed by the method of Rapoport (42).

Glycogen.—When determining this substance in cerebral tissue the writer has found it advantageous to use a separate portion of the pulverized frozen material

only a small fraction of the total ATP of cerebral tissue is active in the glycolytic system.

To overcome the difficulty it is necessary to use stronger precipitating conditions. This was done in the calcium procedure, but in that case fractionation of the hexosephosphates was not attained, as Dr. LePage has pointed out. Modification of procedure A by increasing the barium concentration to 10 times that originally specified gave greatly improved precipitation of the nucleotides and inorganic phosphate, but the separation was still incomplete. Albaum, Tepperman and Bodansky (1) used procedure B as originally described, except that reprecipitation of the barium-insoluble fraction was omitted. The values which they found for acid-labile nucleotides in brains of anesthetized rats may possibly be slightly low, but are approximately in the normal range.

The modified method presented by Dr. LePage uses a considerably greater concentration of barium than was formerly used. Procedure B with this modification might be expected to give satisfactory results in the analysis of cerebral tissue.

These experiences with brain give point to Dr. LePage's caution concerning the indiscriminate use of a fractionation procedure in new experimental circumstances without adequate testing. If a further example is desired, one might compare the results of several different methods used by different investigators for analysis of normal rat liver (10, 20, 28, 43, 44). A number of discrepancies are apparent. Although there is frequent need for a co-ordinated scheme of methods applicable to a single small specimen, the procedure should be checked wherever possible against authenticated methods for individual components. In the barium fractionation it is particularly important that the acid-labile nucleotides be completely precipitated, since their presence in the soluble fraction would interfere with the determination of glucose-1-phosphate, triosephosphate and adenylic acid.

A further difficulty sometimes encountered is the loss of phosphorus compounds by adsorption when barium is precipitated as the sulfate. Such losses may be recognized by comparing the sum of the values for total phosphorus of the three fractions with the total phosphorus in the original extract. Where possible, i.e., in the barium-soluble fractions, such losses might be avoided by removing barium as the carbonate. Another alternative would be to leave the barium in the solutions and to adopt a method for the determination of phosphorus which does not require the presence of sulfate or bisulfite. (Perchloric acid is sometimes substituted for sulfuric in the determination of total phosphorus (4), and various reducing agents are available.)

"True" inorganic phosphorus.—Since ATP is present in the solution, certain precautions are necessary. The determination should be completed within a few hours, since a slow hydrolysis of ATP occurs. This hydrolysis is accelerated by molybdate, to a slight extent in acid solution but greatly in neutral solution. Therefore addition of molybdate should be delayed until ready to proceed with the color development, and at no time should addition of molybdate precede addition of the acid.

Lactic acid.—Conversion to acetaldehyde by heating with sulfuric acid is satisfactory over a wide range of concentration of the acid. However, maximal color intensity is attained if 6.5–9 volumes of concentrated sulfuric acid is used for each volume of aqueous solution (aliquot plus aqueous reagents). In this range, small variations in sulfuric acid concentration do not influence the results.

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when a sufficient quantity is available and to follow the original procedure of Kerr (22) for isolating the glycogen. It is also important to make the correction for nonfermentable reducing material described by Kerr. Presence of a high concentration of sodium chloride in the neutralized hydrolysate must be considered, since it alters the reduction factor in some methods for determining the reducing power.

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